

# A Critical Role of Sodium Flux via the Plasma Membrane $\text{Na}^+/\text{H}^+$ Exchanger SOS1 in the Salt Tolerance of Rice<sup>1[OPEN]</sup>

Houda El Mahi,<sup>a,2</sup> Javier Pérez-Hormaeche,<sup>b,2</sup> Anna De Luca,<sup>b,2</sup> Irene Villalta,<sup>c,i</sup> Joaquín Espartero,<sup>a</sup> Francisco Gámez-Arjona,<sup>d</sup> José Luis Fernández,<sup>e</sup> Mireia Bundó,<sup>f</sup> Imelda Mendoza,<sup>b</sup> Delphine Mieulet,<sup>g</sup> Eric Lalanne,<sup>h</sup> Sang-Yeol Lee,<sup>i</sup> Dae-Jin Yun,<sup>j</sup> Emmanuel Guiderdoni,<sup>g</sup> Manuel Aguilar,<sup>e</sup> Eduardo O. Leidi,<sup>a</sup> José M. Pardo,<sup>b,3</sup> and Francisco J. Quintero<sup>b,3,4</sup>

<sup>a</sup>Instituto de Recursos Naturales y Agrobiología (IRNAS), Consejo Superior de Investigaciones Científicas (CSIC), 41012 Seville, Spain

<sup>b</sup>Instituto de Bioquímica Vegetal y Fotosíntesis (IBVF), Consejo Superior de Investigaciones Científicas (CSIC) and University of Seville, 41092 Seville, Spain

<sup>c</sup>Institut de Recherche sur la Biologie de l'Insecte, Université de Tours, Parc de Grandmont, 37200 Tours, France

<sup>d</sup>Swiss Federal Institute of Technology in Zurich, Zurich CH-8092, Switzerland

<sup>e</sup>Instituto de Investigación y Formación Agraria y Pesquera (IFAPA), Junta de Andalucía, 41200 Seville, Spain

<sup>f</sup>Centre for Research in Agricultural Genomics (CRAG), CSIC-IRTA-UAB-UB, Campus Universitat Autònoma de Barcelona (UAB), 08193 Barcelona, Spain

<sup>g</sup>Centre for International Cooperation on Agricultural Research for Development (CIRAD), Joint Research Unit of Genetic Improvement and Adaptation of Mediterranean and Tropical Plants (UMR-AGAP), 34398 Montpellier, and Université de Montpellier, 34000 Montpellier, France

<sup>h</sup>Reig Jofre, 08970 Barcelona, Spain

<sup>i</sup>Gyeongsang National University, 660-701 Jinju, South Korea

<sup>j</sup>Konkuk University, 05029 Seoul, South Korea

ORCID IDs: 0000-0002-1797-9468 (J.P.); 0000-0001-6220-0372 (D.M.); 0000-0001-5470-5354 (E.L.); 0000-0002-8368-932X (S.L.); 0000-0002-3638-6043 (D.Y.); 0000-0003-2760-2864 (E.G.); 0000-0003-3425-2030 (E.O.L.); 0000-0003-4510-8624 (J.M.P.); 0000-0001-8718-2975 (F.J.Q.).

Rice (*Oryza sativa*) stands among the world's most important crop species. Rice is salt sensitive, and the undue accumulation of sodium ions ( $\text{Na}^+$ ) in shoots has the strongest negative correlation with rice productivity under long-term salinity. The plasma membrane  $\text{Na}^+/\text{H}^+$  exchanger protein Salt Overly Sensitive 1 (SOS1) is the sole  $\text{Na}^+$  efflux transporter that has been genetically characterized to date. Here, the importance of SOS1-facilitated  $\text{Na}^+$  flux in the salt tolerance of rice was analyzed in a reverse-genetics approach. A *sos1* loss-of-function mutant displayed exceptional salt sensitivity that was correlated with excessive  $\text{Na}^+$  intake and impaired  $\text{Na}^+$  loading into the xylem, thus indicating that SOS1 controls net root  $\text{Na}^+$  uptake and long-distance  $\text{Na}^+$  transport to shoots. The acute  $\text{Na}^+$  sensitivity of *sos1* plants at low  $\text{NaCl}$  concentrations allowed analysis of the transcriptional response to sodicity stress without effects of the osmotic stress intrinsic to high-salinity treatments. In contrast with that in the wild type, *sos1* mutant roots displayed preferential down-regulation of stress-related genes in response to salt treatment, despite the greater intensity of stress experienced by the mutant. These results suggest there is impaired stress detection or an inability to mount a comprehensive response to salinity in *sos1*. In summary, the plasma membrane  $\text{Na}^+/\text{H}^+$  exchanger SOS1 plays a major role in the salt tolerance of rice by controlling  $\text{Na}^+$  homeostasis and possibly contributing to the sensing of sodicity stress.

Rice (*Oryza sativa*) is the most important salt-sensitive cereal crop (Hoang et al., 2016). Rice is grown in a diverse range of environments with varying soil-water conditions. Salinity is an intrinsic problem in coastal areas and river deltas where rice farming predominates, but is also a worsening problem in inland areas due to the buildup of secondary salinization of the soil surface as a consequence of excessive use of irrigation with poor quality water and insufficient drainage (Thomson et al., 2010). Excessive amounts of salts in the

soil solution cause substantial declines in the productivity of many agricultural plants, and in rice as little as 3.5 dS/m (2.1 g/L of salts) is sufficient to curtail 10% of yield, and 7.2 dS/m (4.3 g/L) producing 50% yield loss (Munns and Tester, 2008; Hoang et al., 2016).

Salinity poses two main challenges to plants. One is osmotic stress due to excess solutes outside the roots that reduce the ability of plants to extract soil water, which affects cell turgor and expansion immediately. Second, the ionic stress that is most often caused by

excessive influx of sodium ions ( $\text{Na}^+$ ) into the plant leads to interruption of metabolic processes (Hasegawa et al., 2000; Munns and Tester, 2008). Physiological and genetic studies indicate that a range of traits, such as low shoot  $\text{Na}^+$  content, preservation of the  $\text{K}^+$  status of the whole plant, vacuolar compartmentation of salts in older leaves, intrinsic cellular tolerance, and plant vigor, could incrementally improve the ability of rice plants to cope with salinity (Yeo et al., 1990; Thomson et al., 2010). Integration of all these physiological mechanisms for salinity tolerance by pyramiding superior alleles controlling different processes is the long-term goal to increase the level of salt tolerance in rice (Gregorio et al., 2002).

Sustained plant growth under salt stress requires tight control of  $\text{Na}^+$  uptake,  $\text{Na}^+$  redistribution among organs by long-distance transport, and  $\text{Na}^+$  accumulation into cell vacuoles for detoxification and osmotic balance (Munns and Tester, 2008). The ability to manage  $\text{Na}^+$  content has partly a cellular basis since rice protoplasts derived from cultivars with contrasting salt tolerance showed that halotolerance depended on reduced  $\text{Na}^+$  uptake and a fast compartmentation of  $\text{Na}^+$  into vacuoles (Kader and Lindberg, 2005), whereas enhancement of  $\text{Na}^+$  efflux yielded salt-tolerant calli (Zhu and Wu, 2008). At the whole-plant level, comparative studies have shown that salinity-tolerant rice cultivars accumulate less  $\text{Na}^+$  in leaves and shoots compared with that in salinity-sensitive rice cultivars (Golldack et al., 2003; Lee et al., 2003; Lin et al., 2004; Ren et al., 2005) and that shoot  $\text{Na}^+$  accumulation had the strongest negative correlation with the performance of contrasting rice genotypes under long-term salinity (Coskun et al., 2013). Hence, the quantitative measure of the  $\text{Na}^+$  and  $\text{K}^+$  concentrations in the shoot in

salinized conditions has been often used as the trait screened in quantitative trait loci mapping and breeding programs (Hoang et al., 2016). Known proteins governing the partitioning of  $\text{Na}^+$  between roots and shoots are members of the so-called High-affinity Potassium Transporters (HKT), albeit they have repeatedly been shown to behave as  $\text{Na}^+$  transporters mediating either channel-like  $\text{Na}^+$  uniport (class I or HKT1 group) or  $\text{Na}^+$ - $\text{K}^+$  symport (class II or HKT2 group). Generally, dicot species have only a few *HKT* genes in their genomes preferably encoding class-I HKT proteins, whereas monocots have multiple *HKT* genes of both classes. The rice protein OsHKT1;5/SCK1 and the wheat proteins TmHKT1;4/ and TmHKT1;5, all of them class-I proteins, mediate the retrieval of  $\text{Na}^+$  from the xylem sap, thereby restricting the transfer of  $\text{Na}^+$  to shoots. Rice OsHKT1;4 also restricts leaf sheath-to-blade  $\text{Na}^+$  transfer under salinity stress (Cotsaftis et al., 2012; Suzuki et al., 2016).

Although several candidate proteins have been suggested, no major plasma membrane transporters responsible for the bulk influx of  $\text{Na}^+$  into root cells have as yet been conclusively identified at the genetic level, and it seems likely that multiple transport proteins and systems incrementally contribute to  $\text{Na}^+$  influx (Kronzucker and Britto, 2011; Nieves-Cordones et al., 2016). In rice, the HKT2;1 protein that is expressed in the root cortex and endodermis may provide an entry pathway for 'nutritional'  $\text{Na}^+$  uptake under  $\text{K}^+$  limitation to support cell expansion and plant growth (Garcia-deblás et al., 2003; Horie et al., 2007). Sodium may also leak into the root stele of rice plants through anatomical discontinuities in the endodermis produced by the emergence of secondary roots (Yeo et al., 1987), or through the lateral roots themselves (Faiyue et al., 2010a, 2010b). For  $\text{Na}^+$  transport in the opposite (efflux) direction, only the SOS1 transporter (Salt Overly Sensitive 1) has been genetically characterized (Shi et al., 2002; Oh et al., 2009; Olías et al., 2009). In dicots, SOS1 controls net  $\text{Na}^+$  uptake by roots and long-distance transport to shoots (Oh et al., 2009; Olías et al., 2009). Thus, the opposing yet concerted activity of SOS1 in xylem loading and of HKT1-like proteins in xylem unloading might determine the amount of  $\text{Na}^+$  that is eventually exported from roots to shoots. Although HKT proteins of cereal crops are well established to play a major role in their salt tolerance, the contribution of  $\text{Na}^+$  loading into the xylem by specific transporters remains to be determined. Likewise, the regulatory cross talk of these complementary transport systems has not been explored (Ji et al., 2013).

Little is known about the regulation of HKT proteins. *HKT* gene expression is often modulated by  $\text{K}^+$  availability and soil salinity, but the posttranscriptional regulation of HKT transporters remains entirely unknown. In rice, the  $\text{Na}^+$  uptake gene *HKT2;1* is rapidly repressed in roots by as little as 30 mM NaCl to curtail  $\text{Na}^+$  entry (Horie et al., 2007). Surprisingly, *HKT1;4* expression is also down-regulated proportionally to the severity of salinity treatment during the vegetative

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<sup>2</sup>These authors contributed equally to this work.

<sup>3</sup>Senior authors

<sup>4</sup>Author for contact: fjquintero@ibvf.csic.es

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors ([www.plantphysiol.org](http://www.plantphysiol.org)) is: Francisco J. Quintero (fjquintero@ibvf.csic.es).

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growth stage, and RNA interference plants showed no differences in salt sensitivity or in Na<sup>+</sup> content (Suzuki et al., 2016). A contribution of *HKT1;4* toward the prevention of Na<sup>+</sup> accumulation in flag leaf blades was found in the reproductive stage only (Suzuki et al., 2016). *OsHKT1;1*, involved in Na<sup>+</sup> recirculation, is expressed mainly in the phloem of leaf blades and up-regulated by salt stress (Wang et al., 2015). The rice MYB-type transcription factor OsMYBc binds to the *OsHKT1;1* promoter and loss of OsMYBc resulted in a reduction in NaCl-induced expression of *OsHKT1;1* and salt sensitivity.

More progress has been made toward the understanding of SOS1 regulation. The *SOS1* gene is up-regulated by salinity (Shi et al., 2003; Martínez-Atienza et al., 2007) and, in *Arabidopsis* (*Arabidopsis thaliana*), the mRNA is stabilized by an ill-defined process mediated by reactive oxygen species (ROS; Chung et al., 2008). The transport activity of SOS1 is enhanced by protein phosphorylation that counteracts autoinhibition (Quintero et al., 2002, 2011). The minimal SOS functional module in both dicots and monocots comprises proteins SOS1, SOS2/CBL-INTERACTING PROTEIN KINASE24 (CIPK24), and SOS3/CALCINEURIN-B LIKE4 (CBL4; Martínez-Atienza et al., 2007; Quintero et al., 2011). SOS2/CIPK24 is a protein kinase that belongs to the Suc non-fermenting-1-related protein kinase-3 (SnRK3) family. SOS3 is a myristoylated Ca<sup>2+</sup>-binding protein that likely perceives the increase in cytosolic Ca<sup>2+</sup> elicited by excess Na<sup>+</sup> and recruits SOS2 to the plasma membrane to achieve phosphorylation and activation of SOS1 (Quintero et al., 2002, 2011). In *Arabidopsis*, SOS3 functions primarily in roots, whereas the related protein SCaBP8/CBL10 operates by a similar mechanism in shoots (Quan et al., 2007). CBL10 may also regulate Na<sup>+</sup> uptake into the vacuole (Kim et al., 2007).

The rice complement of SOS proteins has been characterized by heterologous expression in yeast and *Arabidopsis* (Martínez-Atienza et al., 2007), but a precise description of their role in the physiology and salinity stress management of rice plants has not been achieved. Here we show that a *sos1* loss-of-function mutant displays exceptional salt sensitivity that correlates with excessive Na<sup>+</sup> intake and impaired Na<sup>+</sup> loading into the xylem. We took advantage of the acute sensitivity of *sos1* plants to Na<sup>+</sup> to inspect the sodicity-stress transcriptome with little interference by the osmotic challenge intrinsic to high salinity treatments. Surprisingly, *sos1* mutant roots showed a marked down-regulation of genes despite the greater intensity of the stress they suffered, which suggested impaired stress detection or an inability to mount a comprehensive response to salinity stress in the absence of SOS1.

## RESULTS

### Selection of Mutant Lines

Public repositories of rice mutant lines were surveyed for entries with putative T-DNA or Tos17

insertions in genes *SOS1* (Os12g44360), *CIPK24/SOS2* (Os06g40370), and *CBL4/SOS3* (Os05g45810). Mutants in the Nipponbare genetic background were preferred because this temperate *japonica* cultivar enabled the lines of interest to complete their life cycle in our experimental paddies. Mutant line codes, annotated insertion points of mutagenic DNA, and insertions experimentally confirmed by diagnostic PCR and sequencing of amplicons are listed in Table 1. Detailed description of the molecular characterization of all insertional lines tested herein is given in the Supplemental Figures S1 to S6. As contrasting materials for mutant phenotyping, congenic plants in which the mutagen was segregated out (null segregants) were kept as controls for subsequent experimentation.

Out of seven mutant lines tested for putative mutagenic insertions at *SOS1* (Piffanelli et al., 2007), only AVIB03 carrying an insertion of transposon Tos17 at intron 19 was confirmed not to produce a wild-type *SOS1* mRNA (Supplemental Figs. S1 to S4). Molecular analyses demonstrated that homozygous plants of this lineage produced a truncated mRNA whose elongation was interrupted by the transposon and that, in contrast with that in other lines (Supplemental Figs. S1 to S4), salinity treatment did not promote the correct processing of the mRNA and eviction of the Tos17 insertion (Fig. 1B). Of note is the significant induction of *SOS1* mRNA by salinity in Nipponbare plants. Sequencing of the reverse transcription (RT)-PCR amplicon with the intervening Tos17 (Supplemental Fig. S4D) demonstrated that transposon insertion in AVIB03 had occurred after nucleotide 9802, relative to the ATG initiation codon in the genomic sequence of *SOS1*. The in silico read-through translation of the putative mRNA predicted the synthesis of a SOS1 protein with a frameshift after residue K767 and the addition of a short C-terminal extension of sequence VCFCFKTTLSH. This truncated protein was predicted inactive based on the removal of essential amino acids and functional domains of the wild-type protein that are downstream the truncation point (Fig. 1A; Quintero et al., 2011; Núñez-Ramírez et al., 2012). To demonstrate this point, a synonymous mutation was created in the rice *SOS1* complementary DNA (cDNA) by introducing a stop codon after residue K767. Expression of this truncated SOS1 protein (SOS1-Δ767) in the yeast (*Saccharomyces cerevisiae*) strain AXT3K that is devoid of all endogenous Na<sup>+</sup> transporters and is salt sensitive (Quintero et al., 2002) failed to restore salt tolerance upon the coexpression of SOS2 and SOS3 proteins of *Arabidopsis* (Fig. 1C). We have shown elsewhere the competence of the *Arabidopsis* SOS2/SOS3 complex to activate the rice SOS1 protein (Martínez-Atienza et al., 2007), and Figure 1C illustrates this fact. By contrast, the rice SOS1 protein with a C-terminal truncation after residue E970 yielded a hyperactive and SOS2/SOS3-independent exchanger (Fig. 1C). Truncation at residue E970 in the rice SOS1 protein mimics the *Arabidopsis* mutant protein SOS1-Δ998, which lacks the autoinhibitory domain of SOS1 that is targeted by the SOS2/SOS3 kinase complex (Quintero et al., 2011).

**Table 1.** Description of rice lines containing *Tos17* or T-DNA insertions in the *SOS* loci

Insertion points (nt, nucleotide) are expressed relative to the ATG initiation codon in the genomic sequences, corresponding to nucleotides 27508321 in *SOS1*, 24043690 in *CIPK24*, and 26535878 in *CBL4*. When confirmed by sequencing of the PCR amplicon containing the mutagenic insertion, the actual insertion point is also given; otherwise it is noted as not determined (n.d.). OTL, *Oryza* Tag Line database; NIAS, National Institute of Agrobiological Sciences, Japan.

Gene	Source	Line Code	Mutagen	Insertion	
				Annotated	Confirmed
<i>SOS1</i>	NIAS	NC2588	<i>Tos17</i>	Intron 5, nt 1228	Intron 5, nt 1228
<i>SOS1</i>	OTL	AVIB03	<i>Tos17</i>	Intron 19, nt 9798	Intron 19, nt 9802
<i>SOS1</i>	OTL	ARBF06	<i>Tos17</i>	Exon 20, nt 9951	No insertion
<i>SOS1</i>	OTL	AKFB09	<i>Tos17</i>	Exon 21, nt 11232	Intron 20, nt 11096
<i>SOS1</i>	OTL	AKFG08	<i>Tos17</i>	Exon 21, nt 11312	Intron 20, nt 11096
<i>SOS1</i>	OTL	AFNB04	<i>Tos17</i>	Exon 21, nt 11402	No insertion
<i>SOS1</i>	OTL	AUJE11	<i>Tos17</i>	Intron 21, nt 11410	Intron 21, nt n.d.
<i>CIPK24</i>	OTL	AMHC11			
		AMHC12	T-DNA	Exon 1, nt -84	Exon 1, nt -84
		AMHE12			
<i>CBL4</i>	OTL	AJBC12	T-DNA	3'-UTR	n.d.

In accordance with the yeast data demonstrating a defective *SOS1* protein in line AVIB03, plants of that line showed a remarkable salt-sensitive phenotype in hydroponic culture that correlated with much greater  $\text{Na}^+$  contents in shoots and roots (Fig. 2). Representative pictures of *sos1* plants at different time points after stress imposition (25, 50, and 75 mM NaCl) are shown in Figure 2A. The *sos1* mutant plants showed growth retardation as early as 5 d after treatment with 50 and 75 mM NaCl. After 10 d, *sos1* plants displayed severe symptoms of stress also at 25 mM NaCl, the lowest salt concentration tested. The experiment was terminated after 24 d when all *sos1* plants had died irrespective of the salt concentration. Quantitative growth data at 7 d after transfer to 50 mM NaCl confirmed the salt sensitivity of homozygous *sos1* plants compared with azygous and wild-type Nipponbare controls (Fig. 2B). At that point, *sos1* roots were severely dehydrated and after 14 d *sos1* shoots had bleached. Salt sensitivity correlated with greater  $\text{Na}^+$  contents in roots and shoots of the *sos1* mutant.

The *SOS1* protein activity accounts for ~60% of the  $\text{Na}^+/\text{H}^+$  exchange measured in plasma membrane vesicles of Arabidopsis (Qiu et al., 2003). Measurement of  $\text{Na}^+/\text{H}^+$  exchange in purified plasma membrane fractions (Supplemental Fig. S5) demonstrated 39% reduction in exchange activity in vesicles derived from the AVIB03 line relative to that in the wild-type plants. Quenching of fluorescence units per mg of protein per minute was  $34.73 \pm 2.56$  and  $56.74 \pm 11.34$ , respectively ( $P = 0.107$  by one-way ANOVA test,  $n = 4$  technical replicas in two different assays). Together, these results signify that line AVIB03 is deficient in *SOS1* activity and hence this lineage was selected for further experimentation.

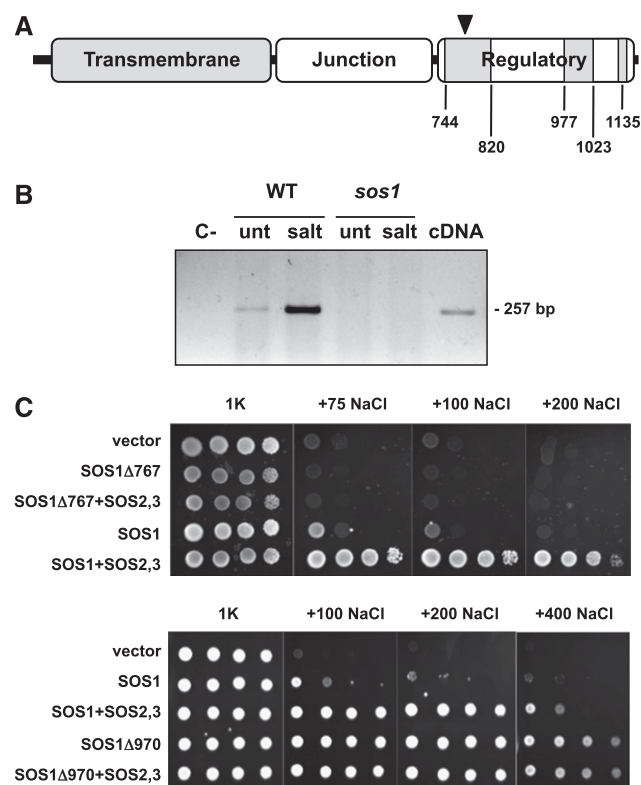
We next searched for mutants in the rice genes *CIPK24* and *CBL4*, which we have previously described as functional homologs of Arabidopsis *SOS2*(*CIPK24*) and *SOS3*(*CBL4*; Martínez-Atienza et al., 2007). Mutant

lines in the Nipponbare background AMHC11, AMHC12, and AMHE12 were annotated to carry T-DNA insertions 84 nucleotides upstream of the translation initiation codon of locus Os06g40370 (*CIPK24*; Sallaud et al., 2004). Molecular characterization confirmed identical T-DNA insertion points at the 5'-untranslated region (UTR) of *CIPK24* in these lines (Supplemental Fig. S6). Homozygous plants produced a mature *CIPK24* mRNA carrying the T-DNA insertion, and no wild-type mRNA was detected. Mutant seedlings were sensitive to NaCl in hydroponic culture. Supplemental Figure S6 illustrates the end-point growth of homozygous *cipk24* plants (L24H), azygous *CIPK24* controls (L24A), and wild-type Nipponbare plants after 7 d in hydroponic medium supplemented with 40 and 80 mM NaCl. These data indicate that the rice kinase *CIPK24*, which has been shown to activate *SOS1* (Martínez-Atienza et al., 2007), also plays a role in the salt tolerance of rice.

At the time this research was conducted, there were no available mutants with mutagenic insertions in the coding region of gene *CBL4* (Os05g45810) in the Nipponbare background. Line AJBC12 was annotated to bear a T-DNA insertion right downstream the 3'-UTR of *CBL4* (Sallaud et al., 2004). RT-PCR analyses with T2 plants of this lineage showed that the *CBL4* transcript was not affected by the adjacent T-DNA insertion, and no further experimentation was done with this line.

### Salt Tolerance Assays in Rice Paddies

To examine in detail the relevance of the *SOS* system in the salt tolerance of cultivated rice, we next sought to define the performance of *sos1* and *cipk24* mutant plants in near-agronomical conditions in experimental rice paddies. Homozygous mutant lines of genotype *sos1* and *cipk24* (denoted henceforth as L1H and L24H, respectively) were compared with congenic segregant lines selected during the genotyping as lacking the



**Figure 1.** Functionality of truncated *SOS1* protein. **A**, Diagram of functional domains of *SOS1*. The junction domain is the C-terminal portion of *SOS1* that has sequence homology with *NHX8* and no known functional role; further downstream is the regulatory domain that is specific and characteristic of *SOS1*-like proteins that has been dissected in three functional modules: the activation domain with sequence similarities to cyclic-nucleotide binding site (amino acids 740–820), the autoinhibitory domain (977–1023), and the CIPK24 phosphorylation site (Ser-1135) that counteracts autoinhibition (Quintero et al., 2011). The arrowhead marks the truncation point after K767 in the mutant protein. **B**, RT-PCR of *SOS1* mRNA of wild-type Nipponbare plants (WT, wild type) and homozygous plants of line AVIB03 (*sos1*), with primers S1-E19-F and S1-E20-R annealing at exons 19 and 20. Detached leaves were treated (salt) or not (unt) with 150 mM NaCl for 24 h. Insertion of Tos17 at the intervening intron 19 prevented the amplification in the *sos1* mutant. As control, the cDNA of *SOS1* was used as template in the PCR reaction (cDNA). **C**, Truncated *SOS1* proteins of rice were expressed in the yeast strain AXT3K, with and without the coexpression of the Arabidopsis *SOS2*–*SOS3* kinase complex, and compared with the wild-type *SOS1*. Decimal dilutions of saturated liquid cultures in selective media were plated in Arginine-Phosphate medium with 1 mM KCl (1K) supplemented with the indicated millimolar concentrations of NaCl.

mutagenic insertion (denoted here as azygous segregant lines L1A and L24A) and to control plants of the Nipponbare background. In lines L1H and L1A the T-DNA of the original transformation was also segregated out. Four-week-old plants were transferred to paddies irrigated with water from an underground well whose salinity content was on average 0.42 g/L, of which  $\text{Na}^+$  was at 5.09 mM,  $\text{K}^+$  at 0.14 mM, and  $\text{Ca}^{2+}$  at 4.0 mM. For the saline treatment, underground water

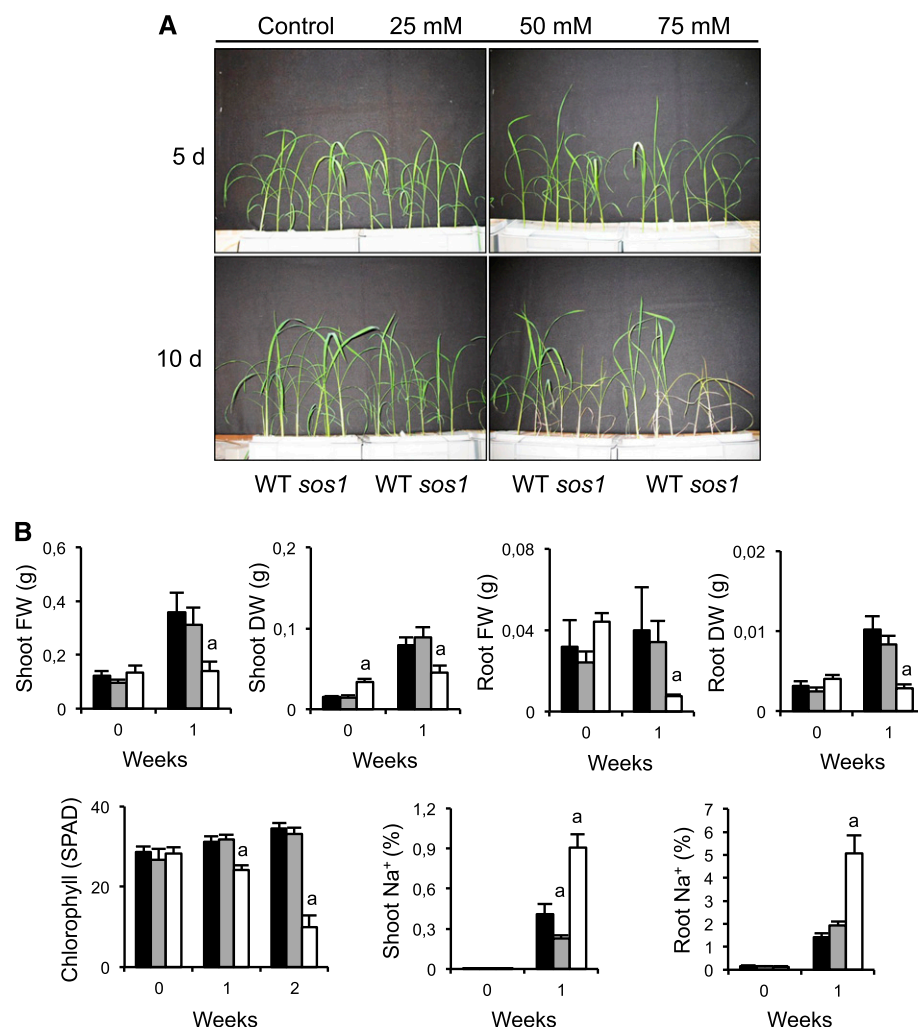
was supplemented with seawater salts to a final concentration of 1.8 g/L salts during irrigation to mimic frequent salinity episodes in rice farms in Spain. The final concentrations of  $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Ca}^{2+}$  in the salinized irrigation water were 27.6, 0.24, and 4.1 mM, respectively. Chloride was 30.5 mM. As depicted in Figure 3 and Supplemental Figure S7, *sos1* plants showed statistically significant growth retardation even at the low salt content of irrigation water, conditions in which the  $\text{Na}^+$  content in *sos1* shoots was low but still triple that of the congenic *SOS1* control and the wild-type Nipponbare (0.035% vs 0.01% of dry matter, respectively). Growth arrest of salinized *sos1* plants at 1.8 g/L salt correlated with massive  $\text{Na}^+$  accumulation in shoots and notable  $\text{K}^+$  loss (Fig. 3). The *cipk24* mutant plants showed a modest growth reduction in these conditions that was statistically significant only at  $P = 0.095$  ( $n = 10$ ) relative to that in the congeneric azygous control (Supplemental Fig. S7), in agreement with the salt sensitivity that mutant *cipk24* showed at early developmental stages in hydroponic culture at 80 mM NaCl but not at 40 mM (Supplemental Fig. S6).

### Complementation and Over-Expression Lines

Because only one putative *sos1* mutant line was available, we sought to confirm that the exceptional salt sensitivity of AVIB03 plants was due to the lack of function of *SOS1* and not to another uncharacterized mutation carried over through the mutant selection process. Genetically complemented lines were obtained by transformation of AVIB03 plants with the *SOS1* cDNA under the control of the constitutive and moderately expressed *UBIQUITIN1* (*UBQ1*) gene promoter of maize. Restoration of salt tolerance was confirmed by scoring the growth of geneticin-resistant T1 plants ( $n = 32$ ), belonging to four independent transformation lines, in hydroponic culture with 50 and 75 mM NaCl for 3 weeks (Supplemental Fig. S8A). This test showed that 13 out of 16 plants regained tolerance to 50 mM NaCl, and that 12 out of 16 withstood the 75 mM NaCl treatment. None of the untransformed *sos1* plants survived the assay ( $n = 8$ ). To further confirm the basis for the suppression of the salt-sensitive phenotype, the complemented line C11 that had a low expression level of the *UBQ1*:*SOS1* transgene (Supplemental Fig. S8B) was chosen for additional analyses. Two-week-old seedlings of wild type, *sos1* mutant, and transformed line C11 were transferred to 25 and 50 mM NaCl in Miyamoto's medium for 3 weeks, after which plant weight, and  $\text{Na}^+$  and  $\text{K}^+$  contents were determined. Results showed that shoot growth in saline media was restored to wild-type levels, whereas root growth lagged behind that of control plants (Fig. 4). Moreover,  $\text{Na}^+$  content in C11 plants largely matched that in wild-type plants.

To test the potential of the *SOS1* exchanger to increase the salt tolerance of rice, we also generated Nipponbare transgenic plants with expression of the





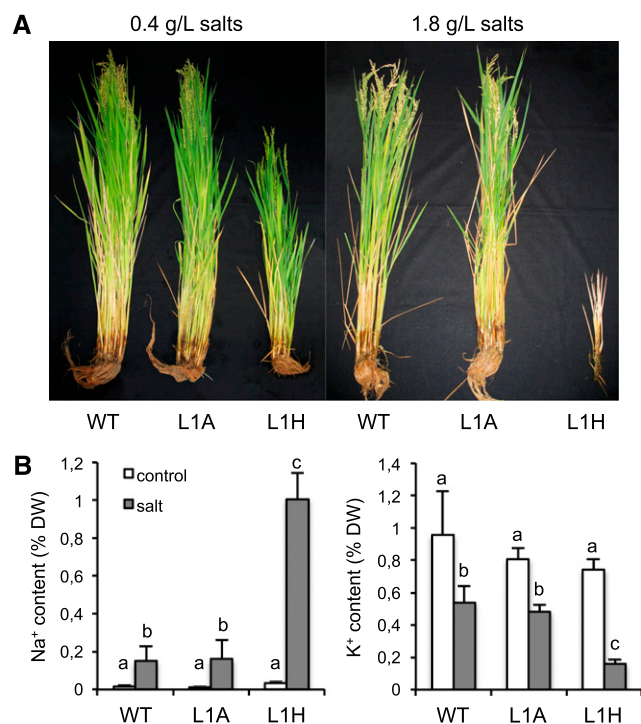
**Figure 2.** Growth parameters of *sos1* mutant in salt. The 5-d-old seedlings of wild type (WT), *sos1* mutant, and a congenic segregant without the *sos1* mutation were transferred to hydroponic culture in Miyamoto's medium supplemented with the indicated amounts of NaCl. A, Depicted are three representative plants per genotype of wild type and *sos1* mutant at 5 and 10 d after transfer to 25, 50, and 75 mM NaCl. B, Fresh weight (FW) and dry weight (DW) of shoot and roots, the chlorophyll content (SPAD units), and the shoot and root Na<sup>+</sup> content of wild type (black columns), *sos1* (white), and the congenic azygous control (gray) before and after 1 week in 50 mM NaCl. The chlorophyll content at 14 d is also given. Plants after 2-week growth in salt were not further processed because roots of the *sos1* mutant were severely damaged. Values are the mean and SD of five plants per genotype. Different letters indicate significantly different values relative to that in the wild type determined by one-way ANOVA ( $\alpha = P < 0.01$ ).

*SOS1* cDNA driven by the strong *Cauliflower mosaic virus* (CaMV) 35S gene promoter to create *SOS1* overexpression. Transgenic plants showing high levels of transgene expression as determined by RT-PCR were selected and propagated to produce homozygous lines. Salt tolerance was evaluated in hydroponic cultures with Miyamoto's medium, which contains low levels of K<sup>+</sup> (0.20 mM) and Na<sup>+</sup> (0.14 mM) and thus could maximize the impact of the ionic component of moderate salt stress (25 to 50 mM NaCl). Seven-d-old seedlings of two independent 35S:*SOS1* lines were cultivated in the presence of 25 and 50 mM NaCl for 4 weeks ( $n = 5$  per genotype and treatment). Transgenic plants overexpressing *SOS1* showed greater survival under salt treatments than that of control plants transformed with a mock empty vector (Fig. 5). Plants that survived the treatment at 25 mM were assessed for dry weight and Na<sup>+</sup> content (control plants surviving 50 mM NaCl had damaged roots and were discarded for further analyses; see Fig. 5A). Plants overexpressing *SOS1* sustained a robust growth at 25 mM NaCl that correlated with lower Na<sup>+</sup> contents in both shoots and roots compared with that in control plants (Fig. 5C), as

should be expected from the near ubiquitous expression of *SOS1* driven by the 35S promoter and the consequently enhanced Na<sup>+</sup> efflux in many cell types.

### Expression Pattern of *SOS1*

In dicots, the *SOS1* protein has been implicated in Na<sup>+</sup> fluxes relevant to net ion uptake by roots and to xylem loading for root/shoot ion partition (Shi et al., 2002; Oh et al., 2009; Olías et al., 2009). To understand the critical role of *SOS1* in the salt tolerance of rice plants, the expression pattern of the *SOS1* gene was investigated in transgenic rice transformed with the *SOS1* gene promoter (defined as the 3.9 kb genomic fragment upstream the initiation codon) fused to the *GUS* reporter gene. Four independent transgenic lines were assayed for *GUS* expression, and they all produced consistent staining patterns, thereby ruling out artifacts by positional effects of the transgene. *GUS* expression was detected at all developmental stages tested, and no significant differences in the expression pattern were found between control and saline



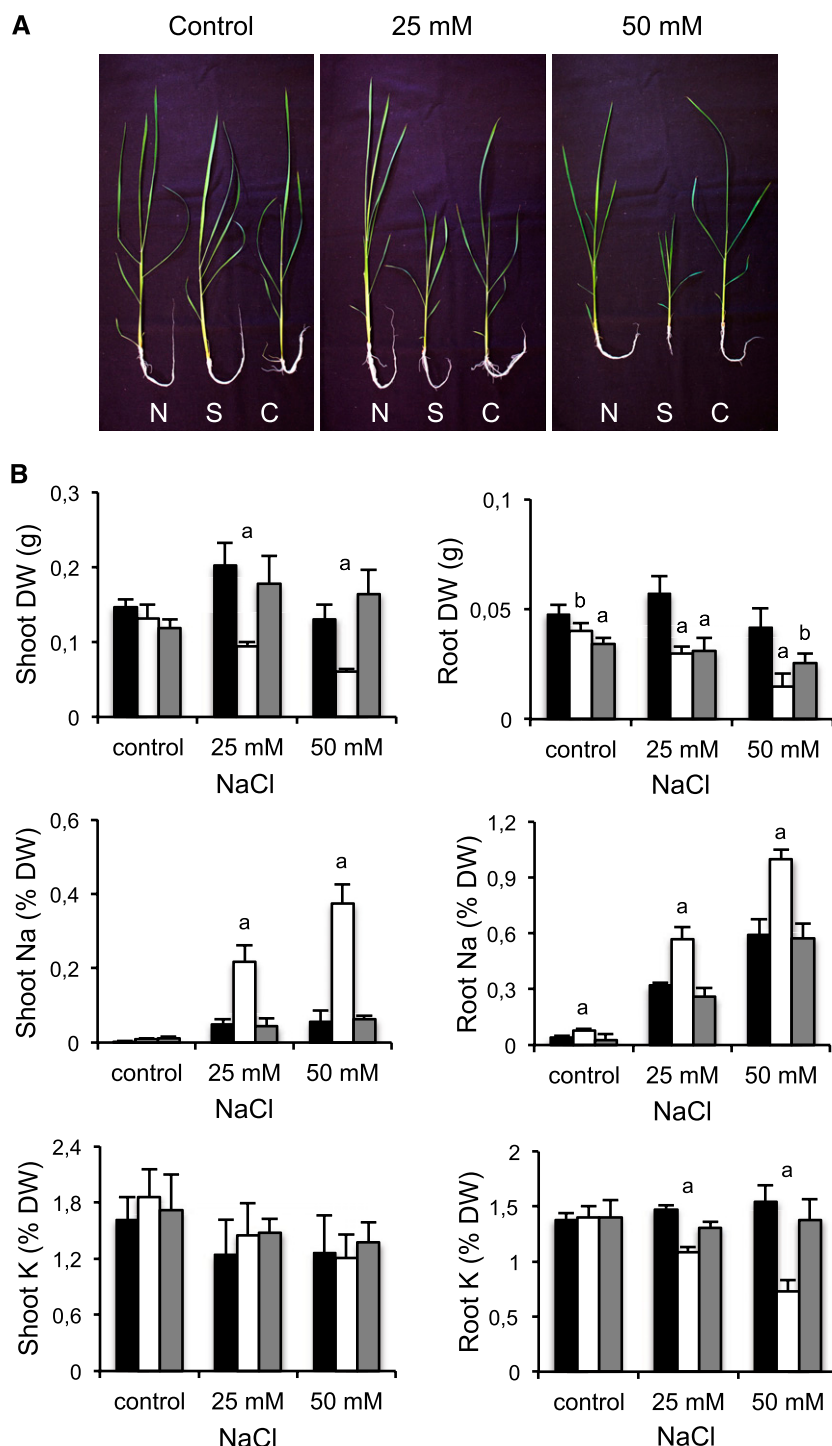
**Figure 3.** Plant growth in experimental paddies. A, Representative plants of *sos1* (L1H), *SOS1* congenic segregant (L1A), and wild type Nipponbare (WT, wild type) genotypes grown for 2 months in experimental paddies irrigated with underground water (left) or supplemented with salt (right). Salt contents in the irrigation water are indicated on top. B, Na<sup>+</sup> and K<sup>+</sup> content in shoots of control (white columns) and salinized plants (gray columns). Genotypes are labeled as in (A). Plotted are the mean and SD of five plants per genotype and condition after 30 d of saline treatment. Different letters indicate significantly different values at  $P < 0.001$  determined by one-way ANOVA. DW, dry weight.

conditions (50 mM NaCl, 24 h). Maximal GUS staining occurred in the vasculature, both in shoots and roots (Fig. 6). In roots, longitudinal and transverse sections indicated a preferential expression in xylem parenchyma (Fig. 6, A–C). No expression was detected at the root apex or root hairs (Fig. 6, D and E). In secondary roots the staining was apparent only after the vascular bundle started to differentiate (Fig. 6, F–H). Likewise, the expression of *SOS1::GUS* was located in vascular bundles in aerial tissues. In the coleoptile, staining was restricted to undifferentiated cells around the two vascular bundles (Fig. 6I). In the flag leaf, all vascular bundles were stained, although the signal was stronger around older xylem vessels (Fig. 6, J and K). There was no detectable expression in stomata (Fig. 6K). Thus, the preferential expression pattern of rice *SOS1* recapitulated the pattern described in *Arabidopsis*, with the exception that the rice reporter gene *SOS1::GUS* was not strongly expressed in root tips as reported for *Arabidopsis* plants using an equivalent approach (Shi et al., 2002). In conclusion, the expression pattern in rice suggested a role for *SOS1* in Na<sup>+</sup> xylem loading in addition to the cellular-based protection imparted by Na<sup>+</sup> efflux.

### Function of *SOS1* in Na<sup>+</sup> Efflux and Xylem Loading

The preferential expression pattern of the *SOS1* gene promoter in parenchyma cells associated to the vasculature suggested a function for *SOS1* in xylem loading since, at physiological Na<sup>+</sup> and H<sup>+</sup> concentrations, a plasma membrane Na<sup>+</sup>/H<sup>+</sup> exchanger likely mediates the energetically uphill Na<sup>+</sup> efflux out of parenchyma cells. Scanning electron microscopy coupled to energy dispersive x-ray microanalysis (SEM-EDX) was used to determine the mineral composition of various root cell types in cross sections of salt-treated rice plants (Fig. 7). In parallel, net tissue contents of Na<sup>+</sup> and K<sup>+</sup> were also determined. Roots of the *sos1* plants treated with 50 mM for 2 d in hydroponic culture had a Na<sup>+</sup> content that doubled the amount in the wild type (112.95 mM vs. 51.4 mM) and showed a 37% reduction in the K<sup>+</sup> content (10.0 mM vs 15.8 mM). In agreement with these values, the amount of Na<sup>+</sup> measured by SEM-EDX in the *sos1* mutant was greater than that in the corresponding wild-type samples of all cell types tested (Fig. 7). In wild-type roots, the maximal Na<sup>+</sup> content was found in the cortex, with a progressive decline in endodermis and xylem parenchyma cells. This profile is in accordance with the selectivity filter imposed by the root endodermis (and buildup of Na<sup>+</sup> content in the cortex relative to that in the endodermis), and with the active loading of Na<sup>+</sup> ions into the xylem vessels after ions entered the stele (minimal Na<sup>+</sup> content in xylem parenchyma). By contrast, the Na<sup>+</sup> distribution in the *sos1* root followed an inward-decreasing gradient signifying the unrestricted transit of Na<sup>+</sup> through all cell layers and the passive leak of Na<sup>+</sup> into the xylem. Together, these results highlight the critical role that the Na<sup>+</sup>/H<sup>+</sup> exchanger *SOS1* plays in Na<sup>+</sup> efflux and xylem loading in rice plants.

On the other hand, the minimal K<sup>+</sup> content in cortical cells in wild-type roots and the increasing K<sup>+</sup> values toward the xylem reflected the active uptake of K<sup>+</sup> by endodermal cells and its translocation toward the vasculature. A key parameter of the physiological status of individual cells and whole plants under salt stress is the Na<sup>+</sup>/K<sup>+</sup> ratio. Figure 7B shows that the ion-selective barrier imposed by the endodermis to the centripetal movement of Na<sup>+</sup> in the root caused a disproportionate Na<sup>+</sup>/K<sup>+</sup> ratio of 11.43 in cortex cells of wild-type roots, in stark contrast with the low 0.49 ratio measured in xylem parenchyma cells presumably effected by the active Na<sup>+</sup> loading in xylem vessels. In accordance with the critical role of *SOS1* in Na<sup>+</sup> export to the xylem, the Na<sup>+</sup>/K<sup>+</sup> ratio in the xylem parenchyma of the *sos1* mutant escalated to 9.40, a 19-fold increase relative to wild-type values. This change in the Na<sup>+</sup>/K<sup>+</sup> ratio between wild type and the *sos1* mutant signified the greatest difference among cell types. These results are also evidence of the profound effects that a deficient Na<sup>+</sup>-specific transport system may produce on the K<sup>+</sup> status of individual cells and tissues.



**Figure 4.** Complementation of *sos1* mutant. The 10-d-old seedlings of wild type, *sos1* mutant, and the complementation line C11 were transferred to hydroponic culture in Miyamoto's medium supplemented with the indicated amounts of NaCl. Plants were harvested 2 weeks later. A, Depicted are representative plants of genotypes Nipponbare (N), the *sos1* mutant (S), and line C11 (C). B, Dry weight (DW) and Na<sup>+</sup> contents in shoots and roots of plants harvested after 7 d of the salinity treatment. Represented are the mean values and SD of wild type (black columns), *sos1* (white), and complementation line C11 (gray) of four plants per genotype and growth condition. Letters indicate significantly different values relative to that in the wild type determined by one-way ANOVA (a =  $P < 0.01$ , b =  $P < 0.05$ ).

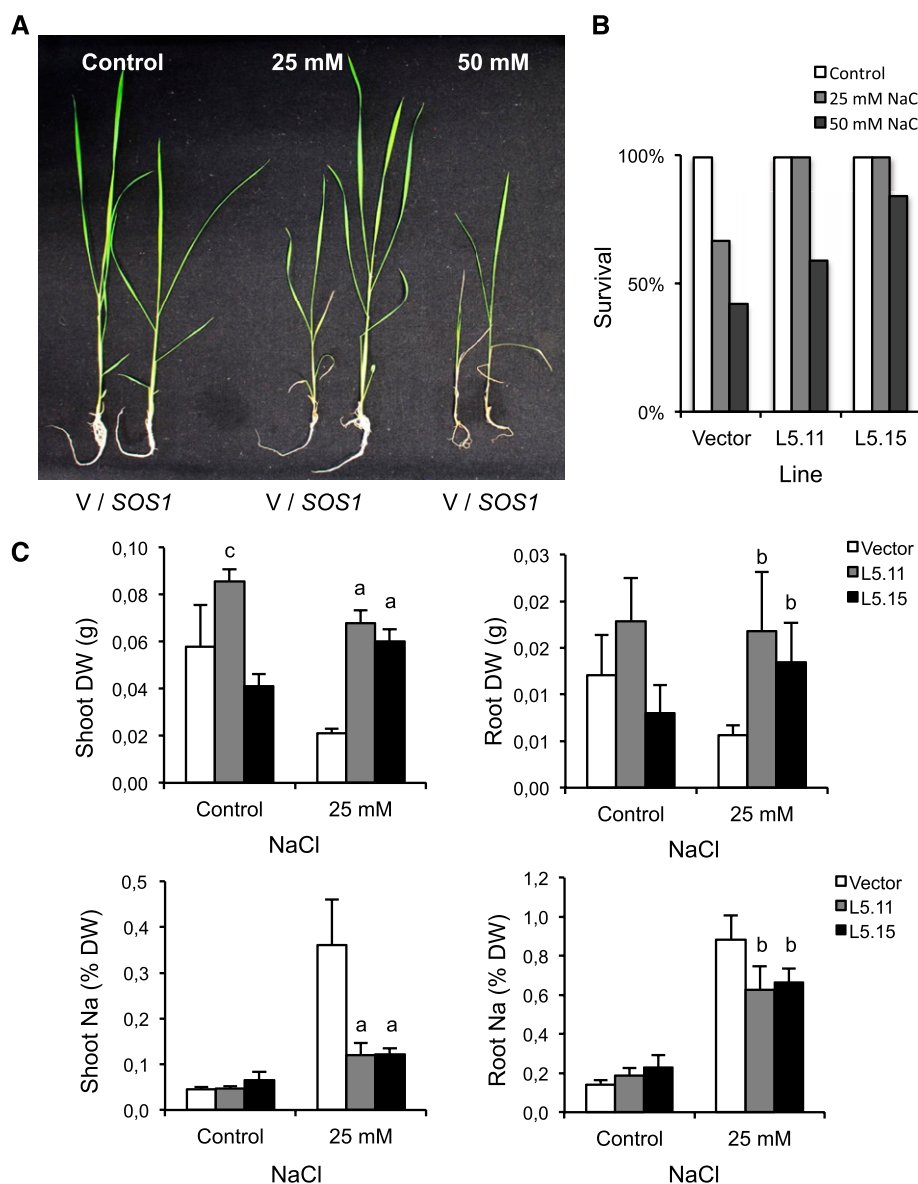
### Transcriptome of the *sos1* Mutant

High salinity imposes both a water stress because the osmotic effect of the saline solution outside and an ionic imbalance resulting primarily from the accumulation of Na<sup>+</sup> and the loss of K<sup>+</sup> (Munns and Tester, 2008). Contrary to most analyses of the transcriptional response of plants to high salinity, in which it is not possible to separate the genetic response to the osmotic

and the ionic components of high salinity (Rabbani et al., 2003; Kanwar et al., 2014; Wang et al., 2016), the extreme Na<sup>+</sup>-sensitivity of the *sos1* mutant plant offered the unique advantage of inspecting the specific transcriptional response to sodicity stress at low external salt concentrations. Roots are the main defensive barrier against salinity and the organ that first senses this soil-derived stress (Munns and Tester, 2008). Hence, to identify genes responding primarily to the Na<sup>+</sup> toxicity



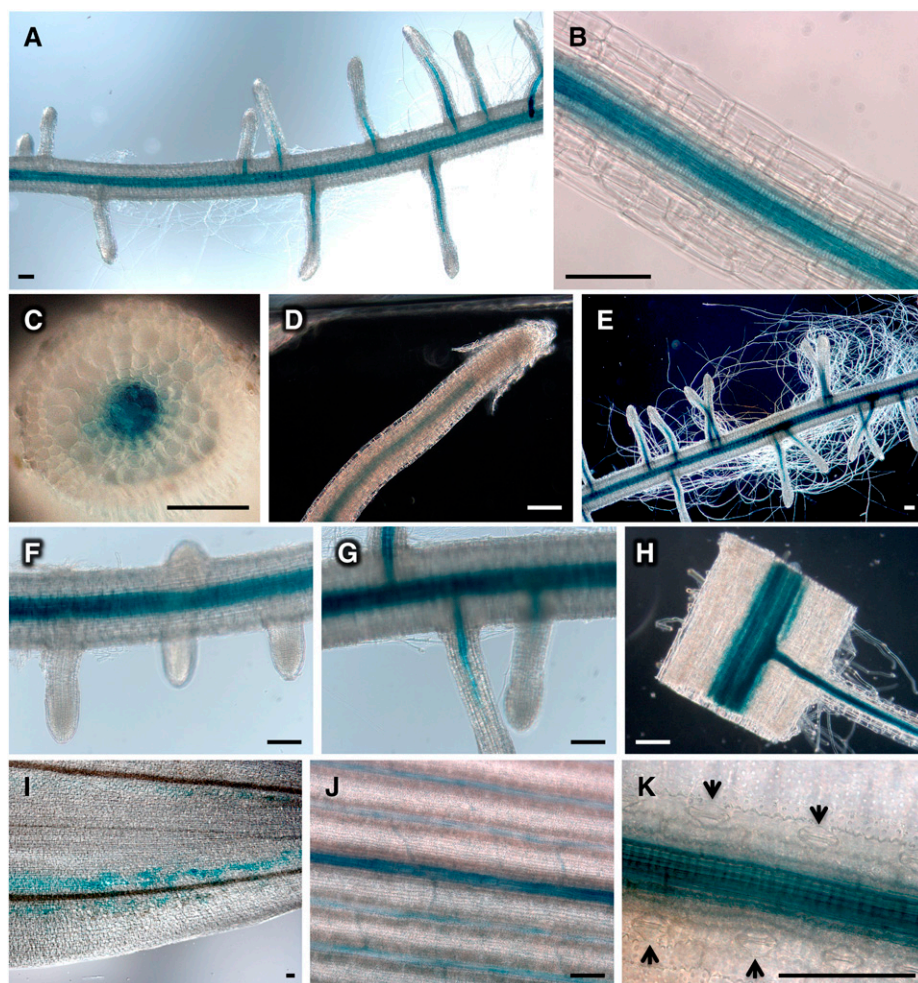
**Figure 5.** Salt tolerance imparted by *SOS1*. The 7-d-old seedlings of two independent Nipponbare transgenic lines expressing *35S:SOS1* were cultivated alongside control seedlings transformed with an empty vector (V) in the absence or presence of 25 and 50 mM NaCl for 4 weeks. A, Representative control (V) and transgenic plants (*SOS1*) are depicted. B, Survival rate of plants after the completion of the experiment. C, Dry weight (DW) and Na<sup>+</sup> content (as percentage of DW) of plants surviving the 25-mM NaCl treatment ( $n = 6$ ). Letters indicate significantly different values relative to that in the wild type determined by one-way ANOVA ( $a = P < 0.01$ ,  $b = P < 0.05$ ,  $c = P < 0.1$ ).



affecting *sos1* plants, roots from mutant and control wild-type seedlings were harvested at the same time point (4 d) after a 75 mM NaCl treatment, and compared with roots harvested alongside from untreated seedlings. Total RNA was extracted and used for microarray experiments (see “Materials and Methods” for details).

Based on the standard criteria of greater than 2-fold changes with statistical significance at  $P < 0.05$  in  $t$  tests, we identified differentially expressed genes (DEGs) in *sos1* mutant roots under salt and control conditions. Supplemental Table S1 online summarizes the genes up- and down-regulated in the mutant roots when compared with expression levels in the control line under the two treatments. Only 84 DEGs, representing 0.3% of total genes annotated, were detected between the two contrasting lines cultivated in control conditions. This result points to a minor role of *SOS1* during the root development in the absence of sodicity stress.

Of note is that *HKT2;1* (Os06g0701700), encoding a transporter protein that mediates Na<sup>+</sup> influx into K<sup>+</sup>-starved roots and thereby improves growth (Horie et al., 2007), was the second most up-regulated DEG in the *sos1* mutant without salinity treatment (Supplemental Table S1). By contrast, many more genes were differentially expressed in the mutant relative to that in the wild type when salt stress was imposed, in accordance with the substantial impact of salinity on *sos1* roots. Of the 5678 total DEGs identified in the roots of the *sos1* mutant under salinity, only 11 genes were commonly up-regulated and 18 were down-regulated in the *sos1* mutant relative to expression levels in the wild type irrespective of the salt treatment (Fig. 8), indicating that the transcriptional impact of the *sos1* mutation was largely specific to the salt-induced response. The large number of DEGs (5633 genes, 19.48% of total) that presented an altered response in *sos1* roots



**Figure 6.** Tissue expression pattern of *SOS1*. A to K, Representative images of staining patterns resulting from GUS expression under the control of the *SOS1* gene promoter. A and B, Preferential expression around the root vascular bundle. C, Root cross section. D and E, Lack of staining in undifferentiated tissues near the root cap and in root hairs. F to H, Staining indicates that expression of *SOS1* correlates with the differentiation of vascular bundles in secondary roots. I, Scattered expression around the vasculature of the coleoptile. J, Vascular bundles of the flag leaf. K, Strong expression in leaf xylem parenchyma. Arrowheads point to nonstained stomata. Scale bars = 100  $\mu$ m.

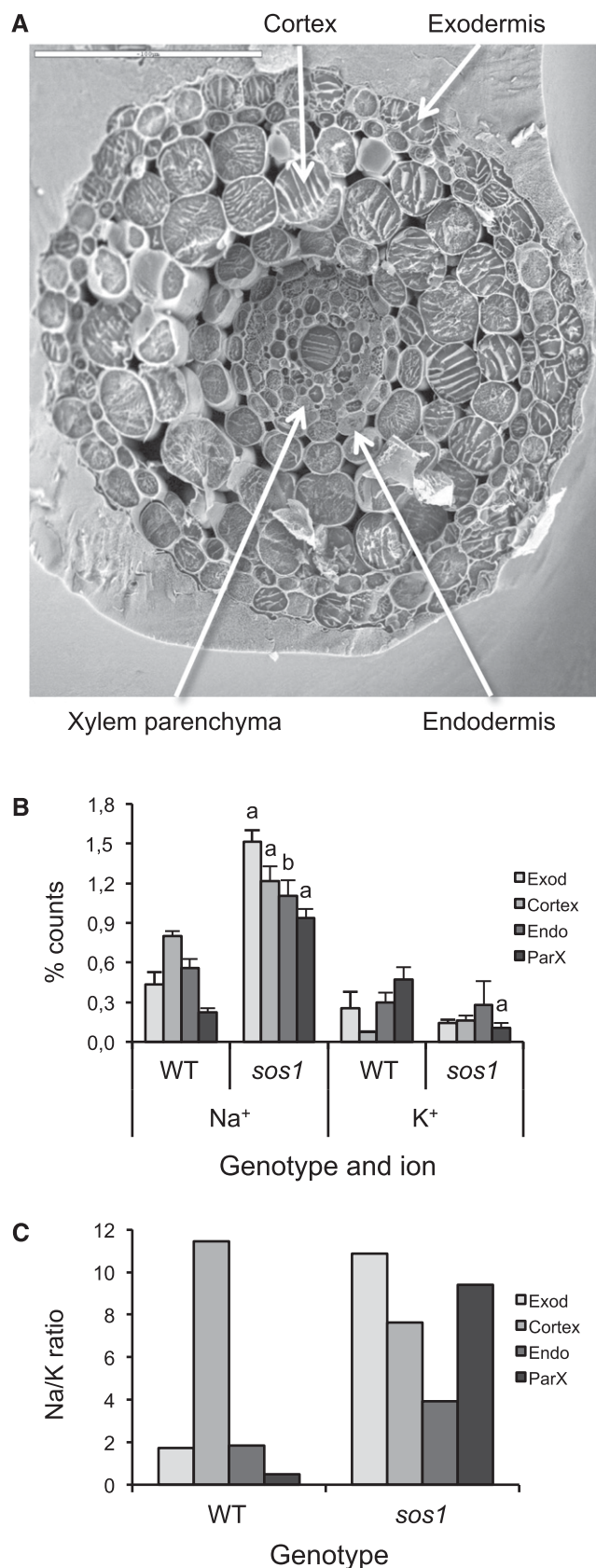
relative to that in wild-type roots under salt stress demonstrates the drastic effect of the *sos1* mutation in the transcriptional response to sodicity. Notably, the majority (64%, 3608 genes) of these salt-responsive DEGs were down-regulated in the mutant root, which may represent a biased transcriptional response toward either repressed or less-induced transcription in the *sos1* mutant in response to salt.

To evaluate the potential biological function of the differentially expressed genes in the *sos1* mutant, Gene Ontology (GO) analysis was performed using the AgriGO service (<http://bioinfo.cau.edu.cn/agriGO>) for the up-regulated, down-regulated, and total DEGs identified in the salt-stressed or control-cultivated roots (Supplemental Table S2). The 5633 salt-responding DEGs fell into 129 significantly enriched GO categories corresponding to 66 biological processes, 28 functional categories, and 35 cellular components. The top over-represented GO categories included “cellular nitrogen compound metabolic process” (137 genes; False Discovery Rate [FDR] of  $7.2 \times 10^{-16}$ ), and those related to the biological process “transport” (FDR =  $2.2 \times 10^{-4}$ ; Supplemental Table S2). Further analysis showed again that these GO categories were constituted

by far more down-regulated than up-regulated genes. This bias explained that most ( $n = 115$ ) of the 129 significant GO terms were also enriched specifically in the group of the down-regulated DEGs (Fig. 8), including the 25 top enriched GO categories (Supplemental Table S3). Moreover, 73 of the significant GO terms identified were only present among the enriched categories of the down-regulated DEGs. For example, among the 97 total DEGs classified under the term “ion transmembrane transporter activity” (GO:0015075), 79 of them were down-regulated DEGs. The GO cellular component “membrane” and “membrane part” ranked top among GO terms enriched in down-regulated DEGs.

The MapMan software (Thimm et al., 2004) was also used to assign genes to functional categories and to further analyze the metabolic pathways and cellular responses altered in *sos1* roots. Over-representation analysis of the up- and down-regulated categories (Supplemental Table S4; Supplemental Fig. S9) indicated a general repression of the glycolysis, the TCA cycle, nitrogen metabolism, amino acid synthesis, redox metabolism, protein targeting (secretory pathway) and protein degradation (ubiquitin proteasome). Among

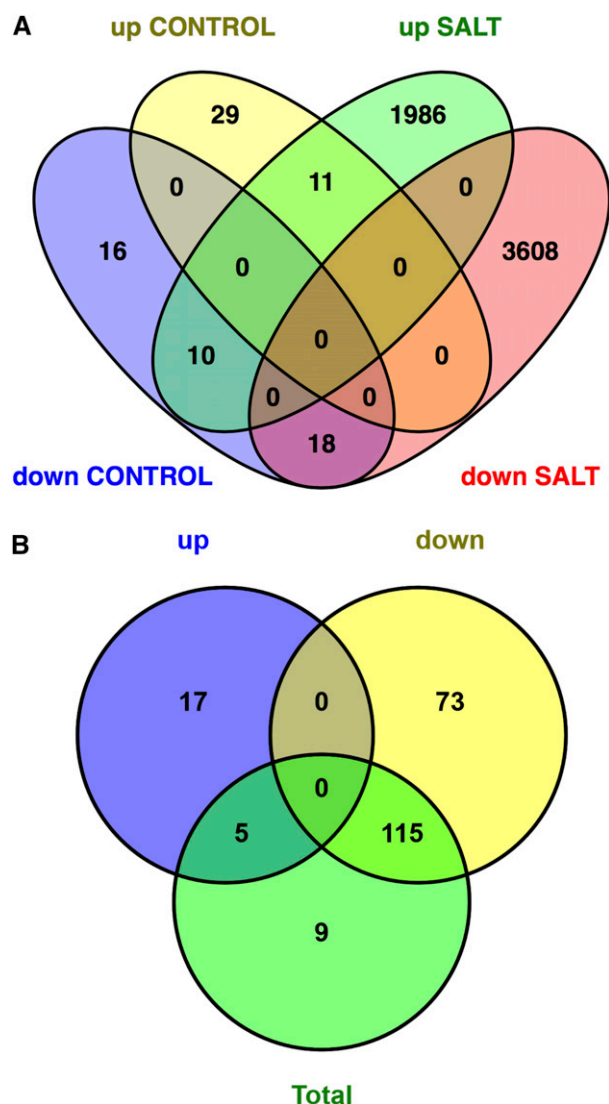




the genes related to “abiotic stress” responses, only the “drought/salt” and “unspecified” subcategories were significantly different between the two genotypes, with an over-representation of unspecified stress genes among the up-regulated salt-responding DEGs. Together, these results support the notion that the transcriptional response of the *sos1* mutant to salt involves a global alteration of the cell physiology due to preferential down-regulation of genes that otherwise would be part of the normal response of wild-type roots to salinity.

The majority of the DEGs showing the largest variations in transcript abundance corresponded to proteins of unknown function or with sequence similarity to other proteins but whose specific function remains to be determined. Moreover, the differential hybridization method used in microarrays only captures genes whose transcript abundance at sampling are substantially different between samples; genes whose transcriptional dynamics are of similar magnitude between samples are not detected even if they responded to the stimulus. This was best exemplified by *CIPK24*, which presumably was not identified as a DEG by the microarray data because this gene was similarly induced in wild-type and *sos1* roots, as demonstrated by RT-quantitative PCR (RT-qPCR; Fig. 9). Thus, we analyzed in greater detail groups of genes encoding proteins with a known role in processes that are likely to be relevant to the ionic component of salinity stress, mainly Na<sup>+</sup> and K<sup>+</sup> transporters and their regulators CIPKs and CBLs. Among the 100 genes selected for this analysis (Supplemental Table S5), the rice *SalT* locus was the most transcriptionally induced gene in *sos1* roots relative to that in control salt-treated roots (log<sub>2</sub> fold change [FC] = 4.79). Gene *SalT*, encoding a 15-kD jacalin-related Man-binding lectin, is located on a quantitative trait loci responsible for the bulk of genetic variation in ion uptake under saline conditions at the seedling stage (Thomson et al., 2010). The strong induction of this gene was confirmed by RT-qPCR (Fig. 9). Likewise, SIMILAR TO RCD-ONE1c (*SRO1c*; Os03g0230300) was highly induced in *sos1* roots in the transcriptomic data set (log<sub>2</sub> FC = 4.56) and verified by RT-qPCR (168-fold induction at 7 d, compared with 2.6-fold in the wild type). *SRO1c* encodes a putative poly-[ADP-ribose] polymerase and is the most stress-responsive gene within the rice *SRO* family (You et al., 2013). Notably, *SRO1c* is closely related to the

**Figure 7.** Mineral content analysis by SEM-EDX. A, Representative SEM cross section of root with labeled arrows indicating the cell types whose elemental composition was analyzed by EDX. Scale bar = 0.1 mm. B, Percentage of total counts of Na<sup>+</sup> and K<sup>+</sup>, measured in 4 to 5 cells of indicated cell types from 2 plants of each genotype (Exod, exodermis; Endo, endodermis; ParX, xylem parenchyma). Plants were treated with 50 mM NaCl for 2 d. Shown are the mean and SD values. Letters indicate significantly different values relative to that in the wild type (WT) for each cellular type determined by one-way ANOVA (a = *P* < 0.01; b = *P* < 0.05). C, Na/K ratios of net content values given in (B). The experiment was repeated twice with similar results.



**Figure 8.** Summary of differentially expressed genes. A, Four-way Venn diagram indicating the number of up-regulated and down-regulated genes found in the comparison between the *sos1* rice mutant and the wild-type Nipponbare in the nonstress-treated (left; Control: 0 mM) and salt-stress (right; Salt: 75 mM)–treated roots. B, Three-way Venn diagram indicating the number of significant GO terms from the gene-ontology analysis of the up-regulated, down-regulated, and total DEGs found in the *sos1* rice mutant and the wild-type Nipponbare in salt-stressed roots.

Arabidopsis protein RADICAL-INDUCED CELL DEATH1 (RCD1), an inactive putative poly-[ADP-ribose] polymerase that shuttles between nucleus and cytoplasm to interact with SOS1 (Katiyar-Agarwal et al., 2006).

Among the CIPK-encoding genes, *CIPK21* showed the largest transcriptional up-regulation by salt in *sos1* vs. wild-type roots according to the microarray data ( $\log_2$  FC = 2.22), which was confirmed by RT-qPCR. A modest 1.93-fold transcriptional induction was measured in wild-type roots after 7 d in salt, whereas in

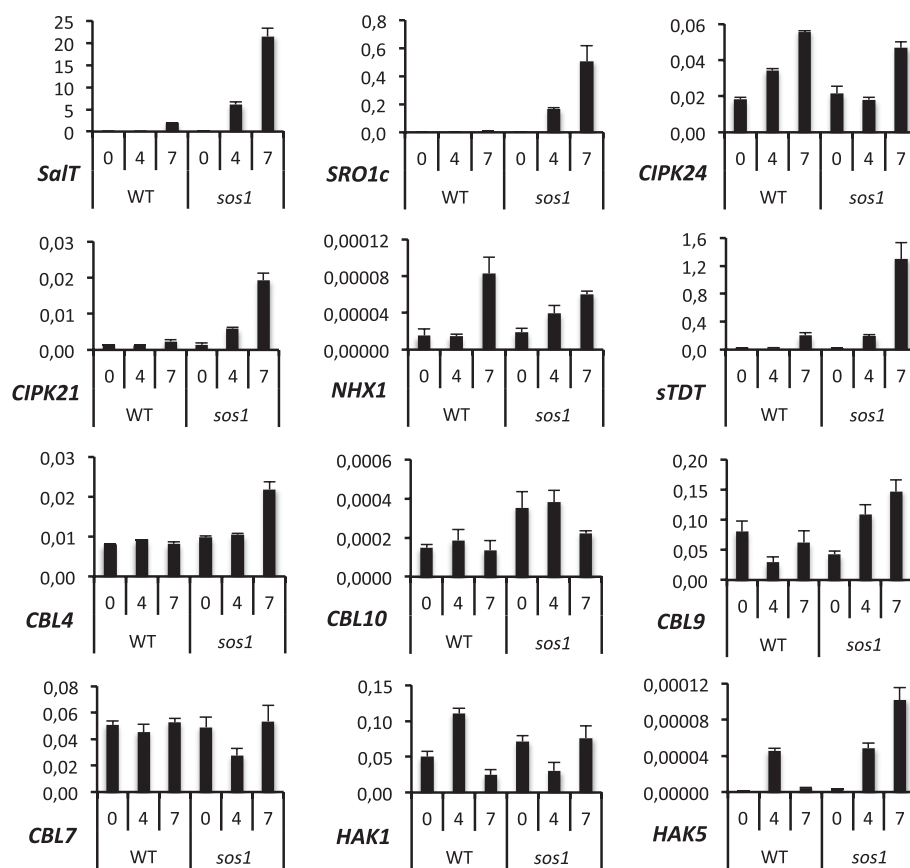
*sos1* the 6.05-fold induction after 4-d treatment was followed by a striking 21.47-fold induction after 7 d (Fig. 9). *CIPK15*, which has been implicated in the salt stress response and enhances the salinity tolerance of transgenic rice plants (Xiang et al., 2007), was also up-regulated in the *sos1* mutant ( $\log_2$  FC = 1.75; Supplemental Table S5). *CIPK1*, 31, 10, and 17 also showed differential up-regulation in *sos1* roots relative to that in the wild type, whereas *CIPK23*, 29, 25, and 4 were down-regulated by at least 2-fold. *CBL* genes showed little differential responsiveness in microarray data, with *CBL9* being the most highly induced ( $\log_2$  FC = 2.04) and *CBL7* the most down-regulated ( $\log_2$  FC = –2.46). RT-qPCR data confirmed the up-regulation of *CBL9* and showed that the down-regulation of *CBL7* in *sos1* was only transient at 4 d, with a return to prestress level after 7 d (Fig. 9). Although not identified as statistically significant DEGs, the rice homologs of *CBL4/SOS3* and *CBL10*, the two *CBLs* regulating *CIPK24/SOS2* and *SOS1* in Arabidopsis, showed complementary kinetics in *sos1* roots so that the transient induction of *OsCBL10* was followed by up-regulation of *OsCBL4* (Fig. 9).

Successful adaptation to salinity requires the accumulation of inorganic and organic osmolytes inside vacuoles for osmoregulation and ion detoxification (Munns and Tester, 2008). Among these osmolytes,  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$ , and organic acids such as malate are most abundant. In Arabidopsis, *NHX1* and *NHX2* are vacuolar  $\text{Na/K/H}$  exchangers that function in planta to drive the uphill accumulation of  $\text{K}^+$  into the vacuolar lumen for osmotic regulation, generation of cellular turgor, and stomatal movements (Leidi et al., 2010; Barragán et al., 2012; Andrés et al., 2014). The rice genes encoding exchangers *OsNHX1* and *OsNHX2* showed a 3- to 4-fold transcriptional up-regulation in *sos1* roots. Gene *Os09g0484900*, encoding a putative protein 75% similar to the tonoplast dicarboxylate transporter (sTDT) of Arabidopsis involved in the uptake of malate into the vacuole (Emmerlich et al., 2003), was also highly induced in the salinized *sos1* roots according to both the microarray and RT-qPCR data (8-fold induction at 7 d in the wild type vs. 85-fold in *sos1*). Malate storage within the vacuole allows the plant to accumulate this metabolite to very high concentrations (up to > 300 mM), and this large gene induction likely reflects the need to accumulate anions in the vacuolar lumen for osmotic adjustment and charge compensation of compartmentalized  $\text{Na}^+$ . These results suggest that in response to salinity, the *sos1* mutant differentially modulates multiple  $\text{K}^+$  and malate transporters to achieve cytosolic ion homeostasis and osmoregulation in intracellular compartments.

Other genes encoding  $\text{K}^+$ -transport proteins showed minor transcriptional responses. No *HIGH-AFFINITY K<sup>+</sup> UPTAKE (HAK)* genes showed differential up-regulation in *sos1* roots compared with that in the wild type according to microarray data. On the other hand, *HAK1*, *HAK5*, and *HAK16* were down-regulated 3- to 4-fold in *sos1* relative to that in the wild type.



**Figure 9.** Quantitation of expression levels of genes relevant to salinity tolerance. RT-qPCR analysis was performed using the  $2^{-\Delta\Delta CT}$  method and the gene expression data normalized for the quantity of *UBQ5* gene transcript before (0) and 4 and 7 d after salt treatment with 75 mM NaCl. All y axes are mean values of normalized expression levels  $\pm$  SD. All x axes are time of treatment and genotype. WT, wild type.



*OsHAK5* mediates high-affinity  $K^+$  uptake by roots under nutrient limitation and in saline conditions (Yang et al., 2014), whereas *OsHAK1* plays a role in  $K^+$  uptake and translocation to shoots over low- and high- $K^+$  concentration ranges (Bañuelos et al., 2002; Chen et al., 2015). Closer inspection by RT-qPCR showed that both *OsHAK1* and *OsHAK5* transcript abundance displayed complex kinetics (Fig. 9). *OsHAK1* transcripts showed opposite kinetics after stress imposition in control and *sos1* plants. A transient induction of *OsHAK5* was also observed in control plants, but the up-regulation of *OsHAK5* was sustained throughout the treatment in the *sos1* mutant. The RT-qPCR data are in agreement with the expected role of *OsHAK5* in sustaining  $K^+$  uptake in a saline environment (Horie et al., 2011; Yang et al., 2014) and probably reflect the need of *sos1* roots to re-establish  $K^+$  homeostasis under salinity.

The HKT proteins of cereals have been involved in the uptake of  $Na^+$  by roots and in the root-to-shoot partition of  $Na^+$  and  $K^+$  (Garcia-deblás et al., 2003; Yao et al., 2010; Munns et al., 2012; Hamamoto et al., 2015). Out of eight HKT genes in the rice genome, only *HKT8/HKT1;5/SHOOT  $K^+$  CONTENT1 (SKC1; Os01t0307500)* showed a modest yet statistically significant down-regulation in *sos1* roots under salt ( $\log_2$  FC =  $-0.81$ ), whereas *HKT1/HKT2;1 (Os06t0701700)* was up-regulated in *sos1* roots in the absence of stress ( $\log_2$  FC =  $2.10$ ). The rice transporter *HKT8/SKC1* regulates  $Na^+/K^+$  levels in

shoots by recirculating  $Na^+$  from shoots to roots (Ren et al., 2005). The significance of the *HKT8/HKT1;5/SKC1* down-regulation might be related to the excessive accumulation of  $Na^+$  in the *sos1* roots and represents a root-protecting mechanism limiting the back-flow of  $Na^+$  to roots.

## DISCUSSION

We showed previously that the rice SOS system comprises the *OsSOS1* transporter, the *OsCIPK24* protein kinase that is the closest homolog to the Arabidopsis *SOS2/AtCIPK24*, and *OsCBL4* that is most similar to *SOS3/AtCBL4* (Martínez-Atienza et al., 2007). The fact that rice *OsSOS1* is encoded by the single copy gene *Os12g44360* allowed a reverse-genetics approach to determine the importance of this protein in the salt tolerance of rice. Here, we have shown that the *sos1* mutant plant displays an exceptional salt sensitivity that is akin to the Arabidopsis *sos1* mutant phenotype. By contrast to the uniqueness of *OsSOS1*, the modest salt sensitivity of the rice *cipk24* mutant relative to *sos1* plants is likely due to functional redundancy. The genetic complement of CIPK-CBL modules in the rice genome comprises 10 CBLs and 33 CIPKs, which is similar to the 10 CBLs and 25 CIPKs known in Arabidopsis (Kolukisaoglu et al., 2004; Gu et al., 2008;

Kanwar et al., 2014). Most of the *CIPK* genes of rice showed transcriptional responses to various abiotic stresses including salinity (Xiang et al., 2007; Kanwar et al., 2014). Because transcription of *CIPK8*, the closest homolog to *CIPK24*, was induced by salt (Xiang et al., 2007; Kanwar et al., 2014), a functional relatedness of *CIPK8* and *CIPK24* appears likely regarding the salt-stress response of rice. Our transcriptomic analysis also flagged *CIPK15*, *CIPK21*, and *CIPK31* as potential players in the sodicity response of roots based on the greater up-regulation in *sos1* roots compared with that in the wild type (Supplemental Table S5). Indeed, *CIPK15* has been reported to increase the salinity tolerance of transgenic rice (Xiang et al., 2007), whereas a loss-of-function *cipk31* mutant was salt sensitive (Piao et al., 2010). There is no reverse genetics data for *CIPK21*, but RT-qPCR confirmed a 21.47-fold up-regulation of *CIPK21* transcripts after 7 d in *sos1* roots (Fig. 8). Likely, this large transcriptional induction of *CIPK21* in the *sos1* mutant reflects its major role in  $\text{Na}^+$  homeostasis. Thus, transcriptomic and genetic data, together with the enormous difference between the salt-sensitive phenotype of the *cipk24* and the *sos1* mutants, suggest that several CIPKs may target *SOS1* for post-transcriptional activation in rice.

Recently, a genetic screen for reduced cesium ( $\text{Cs}^+$ ) accumulation in rice identified a putative loss-of-function allele of *SOS2/CIPK24* as the underlying genetic lesion (Ishikawa et al., 2017). The lower  $\text{Cs}^+$  content in the *lcs1/sos2* mutant correlated with reduced expression of  $\text{K}^+$  transporters *HAK1*, *HAK5*, and *ARABIDOPSIS K<sup>+</sup> TRANSPORT1 (AKT1)* under low external  $\text{K}^+/\text{Na}^+$  ratios, suggesting that  $\text{Cs}^+$  uptake occurred through root  $\text{K}^+$  transporters. Similar to our findings, the *lcs1/sos2* mutant accumulated  $\text{Na}^+$  in roots and was moderately salt sensitive.

### Entry and Redistribution of $\text{Na}^+$

The salt tolerance imparted by *SOS1* is so intimately related to cellular tolerance that overexpression of *SOS1* and resistance to  $\text{NaCl}$  has been used as selection system for rice transformation (Zhu and Wu, 2008). Here, we show that nearly ubiquitous overexpression of *SOS1* driven by the *35S* promoter yielded salt tolerance that correlated with reduced  $\text{Na}^+$  contents in shoots and roots, as should be expected from enhanced efflux of cellular  $\text{Na}^+$  (Fig. 4). In addition, we have shown that *SOS1* also plays a systemic role by redistributing  $\text{Na}^+$  ions between roots and shoots at the xylem-parenchyma boundary (Fig. 6). Rice roots develop Casparian bands and suberin lamellae on the walls of the exodermal and endodermal cell layers that act as physical barriers against the apoplastic flow of water and dissolved minerals (Krishnamurthy et al., 2009). However, lateral roots emerge from the pericycle and disrupt the continuity of the endodermal barrier as they grow toward the cortex and exodermis. This rupture of the apoplastic barrier generates sites through which salts could leak into the main root. The contribution of this 'bypass-flow' to  $\text{Na}^+$

uptake in rice is thought to constitute a major component of the salt sensitivity of this crop, particularly at high transpiration rates (Yeo et al., 1987). Opposing this view, later studies found no evidence indicative of  $\text{Na}^+$  entry at the sites of secondary root emergence in rice plants, although the tissue still remained open to apoplastic flow tracers (Faiyue et al., 2010a, 2010b). Rather, it was concluded that bypass-flow occurred through the lateral roots themselves (Faiyue et al., 2010a). Regardless of whether bypass flow occurs through lateral roots or the endodermal rupture points caused by them, our data support the notion that net  $\text{Na}^+$  fluxes into and across the root are far from being an uncontrolled process. The combination of the tissue expression pattern of the *SOS1* gene promoter and the profile of  $\text{Na}^+$  content along a cross section of rice roots from wild-type and *sos1* mutant plants submitted to a moderate salinity stress (2 d in 50 mM  $\text{NaCl}$ ) indicated that *SOS1* prevented, in the first place, the excessive accumulation of  $\text{Na}^+$  in all cell types of the root and that this protein specifically directed the flow of  $\text{Na}^+$  past the endodermal filter toward the xylem vessels for the acropetal evacuation of  $\text{Na}^+$  out of the root (Figs. 5 and 6). Nevertheless, it should be emphasized that *SOS1* is primarily a cellular-based  $\text{Na}^+$  efflux system (Shi et al., 2002; Oh et al., 2009) and  $\text{Na}^+$  will accumulate over time in root tissues to greater levels in the *sos1* mutant than that in wild-type plants, which would eventually lead to  $\text{Na}^+$  leaking into shoots by mass flow. Moreover, discontinuities in the root endodermis may also allow  $\text{Na}^+$  export out of roots without a biochemical control by dedicated transporters. In other words,  $\text{Na}^+$  will accumulate in the shoot of the *sos1* mutant as a consequence of uncontrolled uptake by roots even if *SOS1* contributes to transporter-mediated  $\text{Na}^+$  loading into the xylem.

The sensitivity of rice plants to salinity is strongly associated with the accumulation of  $\text{Na}^+$  in the leaves (Coskun et al., 2013). Like *SOS1*, the gene encoding the  $\text{Na}^+$ -selective transporter *SKC1/HKT8/HKT1;5* is preferentially expressed in the parenchyma cells surrounding the xylem vessels, where the HKT protein is thought to mediate the recirculation of  $\text{Na}^+$  by unloading  $\text{Na}^+$  from the root xylem (Ren et al., 2005). Our data show that *SOS1* counteracts HKT function by actively exporting  $\text{Na}^+$  from parenchyma cells into the xylem vessels. Thus, the amount of  $\text{Na}^+$  that is eventually delivered to shoots will be determined by the interplay between HKT and *SOS1* proteins acting antagonistically. The optimal set-points for  $\text{Na}^+$  root content and the net transfer of  $\text{Na}^+$  from shoots to roots must be commensurate with the ability of shoot tissues to compartmentalize the incoming  $\text{Na}^+$  to avert ion toxicity while achieving full osmotic adjustment (Pardo, 2010). The down-regulation of *SKC1/HKT8/HKT1;5* in salinized *sos1* roots could represent a root-protecting mechanism limiting the back-flow of  $\text{Na}^+$  to roots that are already exposed to high  $\text{Na}^+$  contents. The reciprocally balanced activity of HKT and *SOS1* proteins has been evidenced also in wheat. Two loci, termed  $\text{Na}^+$  *EXCLUSION1 (Nax1)* and *Nax2* encoding proteins HKT1;4 and HKT1;5, respectively, are genetic

determinants of enhanced  $\text{Na}^+$  retrieval from the xylem and reduced rates of  $\text{Na}^+$  delivery to the shoot (Huang et al., 2006; James et al., 2006; Byrt et al., 2007). Notably, the *Nax* loci also reduced the activity and expression level of the SOS1 exchanger in both root cortical and stele tissues, which contributed further toward reducing the xylem  $\text{Na}^+$  load (Zhu et al., 2016). In Arabidopsis, a functional hierarchy of HKT- and SOS1-dependent transport processes was established by comparing the salt sensitivity of *hkt1* and *sos1-sos3* mutants (Rus et al., 2004). The mutant lacking HKT1 function was sensitive to long-term salinity due to the excessive accumulation of  $\text{Na}^+$  in shoots, and the *hkt1* mutation suppressed the  $\text{NaCl}$  hypersensitivity of the *sos3* mutant by achieving a balanced distribution of  $\text{Na}^+$  that was closer to that of wild-type plants (Rus et al., 2001, 2004). Together, these data demonstrate that a finely regulated interplay between HKT and SOS systems at the xylem parenchyma is a key component of the salt tolerance of vascular plants.

An unresolved question is the mechanistic basis for the intimate relationship of  $\text{Na}^+$ -specific transporters acting at the xylem parenchyma with the  $\text{K}^+$  status of the plant. In rice, both SOS1 and SKC1/HKT8/HKT1;5 selectively transport  $\text{Na}^+$  and yet they affect the accumulation of  $\text{K}^+$  in shoots. Wheat plants carrying the *Nax1* and *Nax2* loci encoding the  $\text{Na}^+$  transporters HKT1;4 and HKT1;5 also had higher rates of  $\text{K}^+$  transport from roots to shoots (James et al., 2006). Root xylem loading is a key step for the delivery of nutrients to the shoot (Drew et al., 1990; Engels and Marschner, 1992; Gaymard et al., 1998). In Arabidopsis, the voltage-dependent  $\text{K}^+$ -channel STELAR  $\text{K}^+$  OUTWARD RECTIFIER (SKOR) mediates xylem loading and root-to-shoot translocation of  $\text{K}^+$  (Gaymard et al., 1998). The SKOR gene is primarily expressed in pericycle and root xylem parenchyma cells. The SKOR channel directs outward  $\text{K}^+$  currents upon membrane depolarization facilitating the release of the  $\text{K}^+$  from the parenchyma cells into the xylem vessels. The Arabidopsis *sos1* mutant was initially described as impaired in  $\text{K}^+$  uptake (Wu et al., 1996), but subsequent research has shown that SOS1 is a highly specific  $\text{Na}^+/\text{H}^+$  exchanger with no ability to transport  $\text{K}^+$  (Qiu et al., 2002; Shi et al., 2002; Quintero et al., 2011). Here we show that removal of SOS1 from its preferential expression tissue at the xylem parenchyma cells leads to the accumulation of  $\text{Na}^+$  ions while in transit to the xylem vessels, with the concomitant reduction in  $\text{K}^+$  content in these same cells. The  $\text{Na}^+/\text{K}^+$  ratio in xylem parenchyma cells climbed from 0.49 in the wild type to 9.40 in the *sos1* mutant, i.e. a 19-fold change (Fig. 6). The coupling of  $\text{Na}^+$  unloading by the xylem-localized HKT transporters with  $\text{K}^+$  loading into xylem vessels via depolarization-activated  $\text{K}^+$  channels has been hypothesized (Horie et al., 2009). HKT-mediated  $\text{Na}^+$  uptake from xylem vessels could induce membrane depolarization of xylem parenchyma cells, which in turn would activate SKOR, the depolarization-activated outward-rectifying  $\text{K}^+$  channel mediating  $\text{K}^+$  release from xylem parenchyma cells. On the other

hand, cytosolic  $\text{Na}^+$  has been shown to inhibit the  $\text{K}^+$  channel AKT1 involved in  $\text{K}^+$  uptake by Arabidopsis roots (Qi and Spalding, 2004). Greater cytosolic  $\text{Na}^+$  concentration in *sos1* plants resulted in impaired  $\text{K}^+$  uptake by roots and poor growth under salt stress. Our data suggest a combined scenario in which the high  $\text{Na}^+/\text{K}^+$  ratio in cortex cells may interfere with root  $\text{K}^+$  uptake and the symplastic movement toward the stele (note that *AKT1* and *AKT2* genes are differentially repressed in *sos1* roots; Supplemental Table S5), whereas the exorbitant  $\text{Na}^+/\text{K}^+$  ratio in xylem parenchyma cells of the *sos1* may interfere with  $\text{K}^+$  loading into the xylem for nutrient partitioning between shoots and roots. This model may also explain the initial observation that the growth of the Arabidopsis *sos1* mutant was impaired under low  $\text{K}^+$  availability. This hypothesis about the mechanistic links between long-distance transport of  $\text{Na}^+$  and  $\text{K}^+$  nutrition deserves further research.

### Transcriptome of the *sos1* Mutant

Nitrate ( $\text{NO}_3^-$ ) is the quantitatively most important anion counter-balancing xylem loading of  $\text{K}^+$  (Engels and Marschner, 1993). In Arabidopsis, the low-affinity, bidirectional  $\text{NO}_3^-$  transporter NITRATE TRANSPORTER1.5 (NRT1.5) mediates  $\text{NO}_3^-$  efflux from pericycle cells to the xylem vessels of Arabidopsis, and SKOR and NRT1.5 cooperate to ensure  $\text{K}^+$  delivery to the shoot via xylem (Drechsler et al., 2015). Alternatively, NRT1.5 could load  $\text{K}^+$  into the xylem directly (Li et al., 2017; Ragel et al., 2019). A similarly complex relationship is also apparent between  $\text{Na}^+$  and  $\text{K}^+$  loading into the xylem (Álvarez-Aragón and Rodríguez-Navarro, 2017). Our transcriptomic data of the *sos1* mutant suggests a broad molecular connection between  $\text{Na}^+/\text{K}^+$  homeostasis and nitrogen metabolism under salinity stress. The biological process, as categorized by GO terms, "Cellular Nitrogen Compound Metabolic Process" was the most severely down-regulated category in the *sos1* mutant under salt relative to that in control roots ( $\text{FDR} = 1.6 \times 10^{-18}$ ). Key proteins involved in nitrate uptake by roots and nitrate assimilation were repressed in the *sos1* mutant. Genes encoding four NRT2-like transporters and two Nitrate Transporter-Activating protein (NAR)2-like regulators have been identified in the rice genome. NAR2.1 interacts with the nitrate transporters OsNRT2.1, OsNRT2.2, and OsNRT2.3a to activate the uptake and distribution of nitrate within the plant (Feng et al., 2011). The strong expression of OsNAR2.1, OsNRT2.1, and OsNRT2.2 in root epidermal cells and that OsNAR2.1 knockdown greatly impaired both high- and low-affinity nitrate transport systems demonstrated the key role of these proteins in nitrate uptake (Yan et al., 2011). Notably, these three key genes were substantially repressed in *sos1* roots by salinity. The  $\log_2$  fold changes in transcript abundance for NAR2.1 (Os02g0595900), NRT2.1 (Os02g0112100), and NRT2.2 (Os02g0112600) were  $-4.24$ ,  $-3.33$ , and  $-2.91$ , respectively. Similarly, the majority of enzymatic genes involved in the nitrate assimilation by its incorporation to

Gln (nitrate and nitrite reductases, and Gln synthetases) were down-regulated in salt-treated *sos1* roots. OsCIKP23 (Os07t0150700), the closest rice homolog to the protein kinase CIKP23 that regulates positively nitrate uptake in Arabidopsis (Ho et al., 2009), was also down-regulated in salinized *sos1* roots relative to that in the control ( $\log_2$  FC =  $-1.66$ ), in agreement with a general repression of nitrogen uptake and metabolism. Consequently, the transcriptional repression of these key genes in the *sos1* mutant root should have a profound effect on nitrate uptake from the mineral medium and subsequent nitrogen assimilation, as already indicated by the GO term and MapMan analyses. The physiological implications of this response are presently unclear, but might be related to the mutual stimulation of nitrate and  $\text{Na}^+$  uptakes that has been observed in various species including rice (Kaburagi et al., 2015; Nie et al., 2015; Gao et al., 2016). In Arabidopsis, nitrate-dependent transport systems mediate the uptake and load of  $\text{Na}^+$  into the xylem, and they may constitute a major pathway for the accumulation of  $\text{Na}^+$  in Arabidopsis shoots (Álvarez-Aragón and Rodríguez-Navarro, 2017). Thus, repression of nitrate uptake by *sos1* roots could have helped to curtail  $\text{Na}^+$  entry and/or distribution.

An intriguing observation is that the GO term 'Response to Stress' was down-regulated in the *sos1* mutant in the two conditions tested, with and without salt, which in principle meant that the *sos1* mutant was less responsive to stress. A number of genes whose function could be reasonably predicted as being beneficial to overcome salinity stress (*CBL4*, *NHX1*, *NHX2*, and the putative vacuolar malate transporter encoded by Os09g0484900) or genes that are known molecular markers of stress intensity (*Salt*) were more highly expressed in the *sos1* mutant than that in wild-type roots. These molecular markers and the severely disturbed  $\text{Na}^+/\text{K}^+$  profiles of *sos1* roots demonstrate that the mutant roots were indeed suffering sodicity stress more intensely than the wild type. Thus, the large proportion of genes down-regulated in the *sos1* mutant might be the consequence of impaired stress detection or an inability to mount a full-fledged response, despite the greater intensity of the stress imposed upon *sos1* roots. Previous studies (Zhu, 2002; Shabala et al., 2005; Ji et al., 2013) have suggested that SOS1 may perform regulatory or sensory functions that may or may not be strictly linked to its function as a  $\text{Na}^+$  transporter. The exceedingly high salt sensitivity of *sos1* mutants of various species (Zhu, 2002; Oh et al., 2009; Olías et al., 2009) and the interaction of the very large C-terminal domain of SOS1 with other stress signaling intermediaries (Katiyar-Agarwal et al., 2006) lend indirect support to this hypothesis. Precedents of this dual function as enzymes and key regulators are the nitrate transporter NRT1.1 and the hexokinase HXK1 that serve as sensor proteins of their substrates (Moore et al., 2003; Ho et al., 2009; Bouguyon et al., 2015).

Alternatively, SOS1 may not be a *bonafide* sensor but serve as a molecular scaffold to facilitate the cross talk of various signaling pathways needed to mount salt tolerance. The SRO proteins are a group of plant-specific

proteins that have important functions in stress adaptation and development. In Arabidopsis, SOS1 interacts with RCD1, a nucleo-cytoplasmic protein that together with AtSRO1 functions to regulate oxidative stress, hormonal, and developmental responses (Katiyar-Agarwal et al., 2006; Jaspers et al., 2010). Moreover, RCD1 interacts with DEHYDRATION RESPONSIVE ELEMENT BINDING2 (DREB2) protein and a number of transcription factors belonging to several protein families. The rice genome contains five SRO genes (*SRO1a* to *1e*), and transcription of *SRO1c* is highly responsive to various stresses including salinity. As in Arabidopsis, rice *SRO1c* has a negative role in resistance to oxidative stress, which seems to be associated to the repression of ROS-scavenging enzyme genes (You et al., 2013). Surprisingly, transcriptomic and RT-qPCR data showed a staggering 55- and 168-fold induction of *SRO1c* in *sos1* roots after 4 and 7 d of salt treatment, which runs counterintuitive to the expected output since ROS scavenging is vital to overcome salinity-induced oxidative stress in rice (Hoang et al., 2016) and *OsSRO1c*-overexpressing rice suffers from greater sensitivity to oxidative stress (You et al., 2013). The observed underrepresentation of DEGs belonging to the GO Biological Process 'Oxidation Reduction' ( $\text{FDR} = 2.3 \times 10^{-6}$ ; Supplemental Table S2) and the GO Molecular Function Oxidoreductase Activity ( $\text{FDR} = 2.6 \times 10^{-5}$ ) in *sos1* roots under salinity are likely related to over-induction of the negative transcription regulator *SRO1c*. Our MapMan analysis of the *sos1* mutant transcriptional response also pointed to a significant enrichment of genes involved in the redox cellular metabolism among the down-regulated DEGs, with 61 down-regulated genes grouped in this category. Detailed analysis of the repressed genes indicates that detoxification enzymes thioredoxins, ascorbate-peroxidases, catalases, and superoxide dismutases were mainly repressed in the mutant relative to that in the wild type. Moreover, further analysis of the mutant transcriptome indicated that the expression of genes grouped in the "drought/salt abiotic stress" category is significantly altered in response to salt (corrected  $p$ -value = 0.037). The differential expression was surprisingly not due to a higher level of induction of the genes of this category in response to salt, but again to a general repression in the *sos1* mutant relative to the wild-type expression level. Thus, it appears that the lack of SOS1 triggers a run-away process leading to unsuitable repression of ROS-controlling genes by *OsSRO1c* and exacerbated salt damage. Taken globally, the transcriptomic profile of the *sos1* mutant root is evidence of an as yet undefined but superior regulatory role of SOS1 in the salinity stress response.

## MATERIALS AND METHODS

### Plant Culture, Salinity Treatments, and Chemical Analyses

Rice seeds were germinated onto wet filter paper for 5 d and then transferred to decapped and perforated Eppendorf tubes prefilled with rockwool. Culture was continued in a hydroponics setting with aeration in modified Miyamoto's



medium (Miyamoto et al., 2001), with weekly transfers to fresh medium. Nutrient medium consisted of 0.09 mM  $(\text{NH}_4)_2\text{SO}_4$ , 0.05 mM  $\text{KH}_2\text{PO}_4$ , 0.05 mM  $\text{KNO}_3$ , 0.03 mM  $\text{K}_2\text{SO}_4$ , 0.06 mM  $\text{Ca}(\text{NO}_3)_2$ , 0.07 mM  $\text{MgSO}_4$ , 0.11 mM Fe-EDTA, 4.6  $\mu\text{M}$   $\text{H}_3\text{BO}_3$ , 1.8  $\mu\text{M}$   $\text{MnSO}_4$ , 0.3  $\mu\text{M}$   $\text{ZnSO}_4$ , 0.3  $\mu\text{M}$   $\text{CuSO}_4$  (pH 5.7), plus 0.3  $\mu\text{M}$   $\text{Na}_2\text{MoO}_4$ . The actual concentrations of  $\text{K}^+$  and  $\text{Na}^+$  in the medium as measured by Atomic Absorption Spectrophotometry were 0.20 mM  $\text{K}^+$  and 0.14 mM  $\text{Na}^+$ . The hydroponic containers were placed in a growth chamber set to a daily light/dark cycle of 16/8 h, photosynthetically active radiation 300  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , 30°C/22°C day/night temperature, and 40–60% relative humidity. Salt treatments were given by transferring the plants to fresh medium supplemented with the indicated amounts of NaCl.

Contrary to Nipponbare plants, seedlings of mutant (L1H and L24H) and congenic azygous lines (L1A and L24A) showed asynchronous germination and heterogeneous growth rates, which presumably arose from somaclonal variation. Therefore, after 1 week in hydroponic culture, outlier plants were removed and only those with sizes similar to Nipponbare controls were transferred to fresh media with and without supplemental NaCl.

For halotolerance tests in experimental paddies in the field, seeds were first germinated in pots with peat. Four-week-old plants of mutant genotypes *sos1* and *cipk24*, their respective congenic null segregants, and of wild-type Nipponbare were then transferred to paddies with clay-loam soil. Plants were arranged in paddies with 5 rows (one per genotype) of 10 plants each, to have 4 repetitions (individual paddies) of each growth condition (control and salinized) and 40 plants per genotype and treatment. Genotypes were arranged differently in each individual paddy. Control paddies were irrigated with water from an underground well with a salt content of 0.4 g/L. Salinity treatment was imposed by automatic mixing of underground water with a salty brine to give a final concentration of 1.8 g/L of seawater salts in the irrigation water.

Chlorophyll contents were determined with a Minolta SPAD-502 Chlorophyll Meter and values are given as SPAD units. To measure  $\text{Na}^+$  and  $\text{K}^+$  contents in tissues, plants exposed to salt stress were separated in shoots and roots and weighed. Roots were washed thoroughly 2 to 3 times over 2 min with cold distilled water to remove surface-contaminating salts, blotted dry, and deep frozen until use. Dried ground material was autoclaved to extract minerals and the supernatant submitted to Atomic Absorption Spectrophotometry.

## Rice Mutant Lines

Nomenclature and genes codes are according to the Rice Genome Annotation Project, version 6.1 (<http://rice.plantbiology.msu.edu/>). Putative mutant lines annotated to carry T-DNA or Tos17 insertions in genes *SOS1* (Os12g44360), *SOS2/CIPK24* (Os06g40370), and *SOS3/CBL4* (Os05g45810) in the Nipponbare genetic background were screened for in the public repositories OryGenesDB of Génoplante (<http://orygenesdb.cirad.fr/>), the Rice Functional Genomic Express Database (<http://signal.salk.edu/cgi-bin/RiceGE>), the Rice Tos17 Insertion Mutant Database (<http://tos.nias.affrc.go.jp/>), and the Rice Mutants Database (<http://rmd.ncpgr.cn/>).

Seeds received from repositories were considered as T2 generation, comprising both heterozygous and homozygous individuals. Plants emerging from these primary T2 seed pools were subjected to diagnostic PCR to identify individuals homozygous for the mutagenic insertion based on the presence of the amplicon predicted from the line annotation and by the absence of the wild-type amplicon when oligonucleotides flanking the insertion point were used as primers in the diagnostic PCR. Primers used for diagnostic PCR and RT-PCR are listed in Supplemental Table S6. Congenic null-segregants without the mutagenic insertion (denoted here as azygous plants) were also selected as controls for further experiments to account for possible epigenetic or phenotypic alterations arising during plant transformation and regeneration procedures. Plants selected after diagnostic PCR were used to produce T3 seeds, and T3 plants were subsequently used for RT-PCR and phenotypic analyses.

## Nucleic Acids, Plasmids, and Transformation

For RT-PCR analyses, total RNA was isolated from approximately 100 mg of frozen plant leaves with TRIZOL (Invitrogen). Synthesis of cDNA with integrated genomic DNA removal was done from 1  $\mu\text{g}$  of total RNA using the QuantiTect Reverse Transcription Kit (Qiagen). PCR was done with DNA purified as described in Murray and Thompson (1980). For sequencing of amplicons and precise identification of insertion points, RT-PCR was performed with Accuzyme (Bioline Reagents Ltd) and amplicons were ligated to vector pCR-Blunt and transformed into *Escherichia coli*. Recombinant plasmids were

purified using the Perfectprep Gel Cleanup kit (Eppendorf), diluted at 100 ng/ $\mu\text{L}$  in water, and sequenced with M13 reverse and forward primers.

Constructs made to test the function of the rice *SOS1* protein in yeast were done in vector pDR195 carrying the *PLASMA MEMBRANE ATPase1* (*PMA1*) gene promoter. Coexpression of Arabidopsis *SOS2* and *SOS3* was achieved with plasmid pFL32T (Quintero et al., 2002). The open reading frame encoding the truncated protein OsSOS1 $\Delta$ 970 was obtained by PCR using as template the wild-type cDNA (Martínez-Atienza et al., 2007) and with oligonucleotides that included *Bam*HI cutting sites and a stop codon after amino acid L969 of *SOS1*. The resulting amplicon was digested with *Bam*HI and inserted in pDR195. Yeast transformation followed the Lithium-Acetate method. The *Saccharomyces cerevisiae* strain AXT3K ( $\Delta\text{ena1::HIS3::\textit{ena4}}$ ,  $\Delta\text{aha1::LEU2}$ ,  $\Delta\text{nhx1::KanMX4}$ ) has been described elsewhere (Guo et al., 2004). Sodium tolerance tests were performed in the alkali cation-free Arginine-Phosphate medium (Rodríguez-Navarro and Ramos, 1984) supplemented with 1 mM KCl and with NaCl as indicated in each experiment.

To determine the expression pattern of *SOS1*, a 3.9-kb fragment in the intergenic region between *SOS1* (Os12g44360) and the adjacent gene (Os12g44370) and proximal to the coding region of *SOS1* was amplified by PCR and inserted as a *Sall* and *Xba*I fragment into vector pBIG containing a promoter-less *GUS* reporter gene (Becker, 1990). *GUS* chemical staining with X-gal in whole tissues was as described in Jefferson et al. (1987). To test complementation of the *sos1* mutant, the full-length cDNA of rice *SOS1* was cloned in a pCambia1300-derived vector containing the maize *UBIQUITIN1* gene promoter and the *nopaline synthase* terminator (Campo et al., 2014). For *SOS1* overexpression, the cDNA was cloned into the vector pCambia2300 under the control of the CaMV 35S promoter and terminator and transformed in Nipponbare plants. Rice transformation by *Agrobacterium tumefaciens* EHA105 has been described (Campo et al., 2014).

## Measurement of $\text{Na}^+/\text{H}^+$ Exchange in Membrane Vesicles

Plasma membrane vesicles were isolated from 2-month-old rice roots from 24-h salinized (50 mM NaCl) plants using two-phase partitioning as described by Qiu et al. (2003). All steps were carried out at 4°C or on ice. Roots were homogenized in buffer containing 0.33 M Suc, 10% (w/v) glycerol, 0.2% (w/v) bovine serum albumin, 5 mM EDTA, 5 mM dithiothreitol (DTT), 5 mM ascorbate, 0.2% (w/v) casein, 0.6% (w/v) polyvinylpyrrolidone, 1 mM phenylmethylsulfonyl fluoride, 1  $\mu\text{g}/\text{mL}$  pepstatin A, and 50 mM HEPES-KOH (pH 7.5). Eight milliliters of homogenization buffer were used per gram of tissue. The homogenate was filtered through two layers of Miracloth (Merck) and centrifuged at 10,000 g for 10 min. The supernatant then was centrifuged for 50 min at 100,000 g to obtain a microsomal pellet that was resuspended in a buffer containing 0.33 M Suc, 3 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1  $\mu\text{g}/\text{mL}$  pepstatin A, and 5 mM potassium phosphate (pH 7.8). The suspension was added to a phase mixture to obtain a phase system consisting of 6.2% (w/w) Dextran T-500 and 6.2% (w/w) polyethylene glycol 3350 in 5 mM potassium phosphate (pH 7.8), 0.33 M Suc, and 3 mM KCl. Phase separation was facilitated by centrifugation at 1,000 g. The upper phase was then repartitioned twice in this system. The final upper phase was collected, diluted with suspension buffer [0.33 M Suc, 10% (w/v) glycerol, 0.1 mM EDTA, 2 mM DTT, 1  $\mu\text{g}/\text{mL}$  pepstatin A, 20 mM HEPES-KOH (pH 7.5)], and centrifuged for 45 min at 100,000 g. The resulting pellet was collected and resuspended with the above-described suspension buffer containing 1 mM EDTA, frozen in liquid nitrogen, and stored at  $-80^\circ\text{C}$  until use. Before membrane isolation, plants were treated for 24 h with 50 mM NaCl in Miyamoto's medium to induce *SOS1* activity. Vesicles purified from nontreated plants produced very low rates of  $\text{Na}^+/\text{H}^+$  antiport activity, in agreement with previous reports (Qiu et al., 2003).

The measurement of  $\text{Na}^+/\text{H}^+$  proton exchange was as described by Qiu et al. (2003), except that fluorescence quenching of 9-amino-6-chloro-2-methoxy-acridine was used to monitor the formation and dissipation of pH gradients. Fluorescence was recorded with a Hitachi fluorescence spectrophotometer (FL-2500) in a thermostated cell (25°C) at excitation and emission wavelengths of 415 and 485 nm, respectively (slit-width, 10 nm). Purified plasma membranes (50  $\mu\text{g}$  of protein) were added to a buffer containing 250 mM mannitol, 10 mM bistris-propane-MES (pH 6.8), 100 mM KCl, 3 mM  $\text{MgSO}_4$ , and 1  $\mu\text{M}$  9-amino-6-chloro-2-methoxy-acridine (1 mL final volume). The  $\Delta\text{pH}$  was generated with the activity of the plasma membrane  $\text{H}^+$ -ATPase and recorded as fluorescence quenching after ATP addition (2.5 mM ATP, pH 6.8). The  $\text{Na}^+$ -induced dissipation of  $\Delta\text{pH}$  was initiated after adding 100 mM NaCl into the cuvette, and recorded as the recovery of fluorescence. Exchange rates are calculated as fluorescence recovery relative to that before ATP addition ( $\Delta\text{F}/\text{F}_{\text{max}}$ ) per minute and milligram of protein.

## Microarray Analysis

Control and *sos1* seedlings were grown hydroponically in Miyamoto's medium for 3 weeks in environmentally controlled conditions (12 h of light at 25°C and ~450  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ , and 12 h darkness at 21°C; relative humidity approximately 65%). Salt stress was imposed by transferring to fresh nutrient solution containing 75 mM NaCl for 4 d. Control plants received fresh nutrient solution without NaCl. No signs of plant deterioration were observed at the end of the salinity treatment. Total RNA was extracted from whole roots pooled from five plants per genotype and treatment. The quality and concentration of the RNA were determined using the Agilent 2100 Bioanalyzer, and samples with an RNA Integrity Number less than 6 were discarded. Three on-chip technical replicates per biological sample were analyzed. Gene expression profiling was performed by microarray hybridization using a custom rice transcriptomic array (Oryzon Genomics) containing  $3 \times 29450$  probes (3 technical replicates per probe) representing 20750 genes. Microarray construction, probe labeling, and hybridization conditions were as in Campo et al. (2014). Results were normalized using self-to-self comparison Nipponbare vs Nipponbare, and fold change was calculated as  $\log_2$  of the ratio of relative expression of *sos1* (sample, Smp) versus wild-type (control, Ctr) plants grown in identical conditions. Only genes for which replicate spots showed differential expression of  $-0.5 > \log_2(\text{Smp/Ctr}) > 0.5$  and  $P$  value  $< 0.05$  were considered as differentially expressed. Bootstrap analysis with Significance Analysis of Microarrays was used to identify differentially expressed genes using a cutoff of 2 (Tusher et al., 2001). Significance Analysis of Microarrays calculates the fold change and significance of differences in expression. Data sets for microarray analyses have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus with accession number GSE129844.

Differential expression of a select number of DEGs was confirmed by reverse-transcription-quantitative PCR (RT-qPCR). Three-week-old control and *sos1* seedlings were treated with 75 mM NaCl in Miyamoto's medium for 4 and 7 d. Total RNA was extracted from roots, and 500 ng were used to synthesize the cDNA using the Quantitect Reverse Transcription kit (Qiagen). Quantitative real-time PCR assays were performed using the CFX Connect Real-Time PCR Detection System (Bio-Rad). The PCR mixture (total volume of 20  $\mu\text{l}$ ) was composed of 5  $\mu\text{l}$  of diluted cDNA, 10  $\mu\text{l}$  iTaq Universal SYBR Green supermix (2 $\times$ ), 0.3  $\mu\text{M}$  of each primer, and water. The specific oligonucleotides used are listed in the Supplemental Table S6. Thermal cycling consisted of 95°C for 3 min, followed by 40 cycles of 10 s at 95°C, 15 s at 58°C, and 15 s at 72°C. A melting curve was generated to check the specificity of the amplified fragment. The efficiency of all primers under these conditions was 85–100% for all samples tested. Rice *UBIQUITIN5* was used as a house-keeping gene in expression analysis. Relative gene expression was determined using the  $2^{-\Delta\Delta\text{CT}}$  method (Livak and Schmittgen, 2001). Threshold cycle values were determined using ICYCLER Q software (Bio-Rad).

## Statistical Analyses

Numerical data were analyzed with IBM SPSS Statistics v.21 (Armonk) using a completely randomized design model. Means and variances were compared by one-way ANOVA to find significant differences using the Fisher's Least Significant Differences test at  $P < 0.01$  level, unless stated otherwise.

## Accession Numbers

Sequence data from this article can be found in the Rice Genome Annotation Project (<http://rice.plantbiology.msu.edu/>) and UniProt (<https://www.uniprot.org/>) databases under accession numbers *SOS1* (LOC\_Os12g44360, Q5ICN3), *CIPK24/SOS2* (LOC\_Os06g40370, Q69Q47), and *CBL4/SOS3* (LOC\_Os05g45810, Q75KU4).

## Supplemental Data

The following supplemental information is available:

**Supplemental Figure S1.** Molecular characterization of rice lines AKFG08 and AKFB09.

**Supplemental Figure S2.** Molecular characterization of rice line NC2588.

**Supplemental Figure S3.** Molecular characterization of rice line AUJE11.

**Supplemental Figure S4.** Molecular characterization of the rice line AVIB03.

**Supplemental Figure S5.**  $\text{Na}^+/\text{H}^+$  exchange activity in plasma membrane vesicles.

**Supplemental Figure S6.** Molecular characterization of the rice lines AMHC11 and AMHC12.

**Supplemental Figure S7.** Salinity tolerance test in experimental paddies.

**Supplemental Figure S8.** Complementation of the *sos1* mutant.

**Supplemental Figure S9.** MapMan analysis of the metabolic and cellular responses of *sos1* roots to salt.

**Supplemental Table S1.** Differentially expressed genes (DEGs) in the *sos1* mutant roots when compared to the control line (Excel file).

**Supplemental Table S2.** Gene Ontology (GO) analysis of total DEGs identified in the salt-stressed or control-cultivated roots (Excel file).

**Supplemental Table S3.** Top 25 enriched GO categories (Excel file).

**Supplemental Table S4.** MapMan analysis of DEGs identified in the salt-stressed *sos1* roots (Excel file).

**Supplemental Table S5.** Differential expression levels of select genes involved in  $\text{Na}^+$  and  $\text{K}^+$  transport processes (Excel file).

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